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# ANNALES MEDICINAE EXPERIMENTALIS ET BIOLOGIAE FENNIAE

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REDIGENDOS CURAVIT

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VOL. 32

1954

FASC. 2

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*Annales Medicinae Experimentalis et Biologiae Fenniae*

Yrjönkatu 17, Helsinki, Finland.

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## EFFECTS OF MERCURIAL DIURETICS IN RAT KIDNEY

### HISTOCHEMICAL STUDIES

by

KIMMO K. MUSTAKALLIO and ANTTI TELKKÄ<sup>1</sup>

(Received for publication December 30, 1953)

Pitts and Sartorius (19) concluded in their extensive review of diuretics that the site of the direct tubular action of mercurial diuretics is unknown. Recently Grossman (11) emphasized that many aspects of the activity of mercurials on a cellular level still require a detailed study. The attempts made to localize the site of the tubular reabsorptive depression are based on indirect evidence principally from toxicologic and excretion studies.

In the proximal tubule, water and electrolytes are reabsorbed as an isosmotic solution while in the distal tubule water can be reabsorbed against an osmotic gradient, a process requiring energy. This latter process, the facultative reabsorption, the mechanism of which is unknown, probably gathers its energy from the oxidative metabolism of carbohydrates. According to Barron (5, 6) the dehydrogenases involved in the citric acid cycle of Krebs require sulfhydryl (-SH) groups for their normal activity. Furthermore, the mercury-containing compounds are strong inhibitors of these enzymes by combining with their essential -SH groups. That the diuretic effect of mercurials is attributable to the depression of the sulfhydryl-requiring enzymes seems probable since it is abolished by the administration of dimercaptopropanol (BAL) containing two -SH groups (19). Handley and Lavik (14) found, by determining

<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

the  $O_2$ -uptake of kidney cortex homogenates, that the administration of mercurial diuretics to rats significantly depresses the succinic dehydrogenase activity. This -SH-requiring enzyme is an essential link in the citric acid cycle of Krebs catalyzing the dehydrogenation of succinic acid to fumaric acid.

The purpose of this investigation is by employing histochemical methods, principally for sulfhydryl groups and succinic dehydrogenase, to approach the mode of action of mercurial diuretics within the renal tubule of rat.

#### THE PRESENT INVESTIGATION

90 albino rats of Wistar strain, weighing 175—225 g were used. 30 of them served as controls and 10 for toxicity determination. Novurit »Medica» (mercurophylline, 39.5% Hg) was administered subcutaneously or intramuscularly in doses of 10—50 mg Hg/kg of body weight. The rats were killed by decapitation 2—6 hours after the administration of Novurit.

For histochemical demonstration of protein-bound sulfhydryl groups the method of Barnett and Seligman (3) was followed. The reagent, 2,2'-dihydroxy-6,6'-dinaphtyl disulfide (DDD)<sup>1</sup>, when used at pH 8.5, reacts with active -SH groups of fixed tissue proteins to form a colourless substance, which is converted into a coloured azo dye by coupling with tetrazotized diorthoanisidine. Furthermore, unfixed frozen sections were treated with the nitroprusside method according to the modifications of Bourne (7) and Hammett and Chapman (13), and with the ferric-ferricyanide reduction test according to Chèvremont and Frédéric (8) and Lillie and Burtner (15).

Succinic dehydrogenase activity was demonstrated histochemically employing the method of Seligman and Rutenburg (21). The chemical reaction involved consists of oxidation of succinic acid to fumaric acid and reduction of a soluble tetrazolium salt to insoluble coloured formazan. In this reaction system, tetrazolium salts serve, instead of natural cytochromes, as hydrogen acceptors for succinic dehydrogenase. Three tetrazolium salts were used, blue tetrazolium (BT)<sup>2</sup>, 3,3-dianisole bis- 4,4 (3,4 diphenyl) tetrazolium

<sup>1</sup> Obtained from Schwarz Laboratories, Inc., Mount Vernon, N. Y.

<sup>2</sup> Obtained from the Dajac Laboratories, Monomer Polymer, Inc., Chicago, Ill.

chloride (21), neotetrazolium (NT)<sup>1</sup>, pp'diphenylene-bis-2-(3,5 diphenyl) tetrazolium chloride (1,22) and 2,3,5 triphenyl tetrazolium chloride (TPT)<sup>2</sup> (2). Some of the sections were treated in anaerobic conditions and with ionic activators according to Padykula (17) and Rutenburg, Wolman and Seligman (20). The specificity for succinic dehydrogenase was tested using  $2.5 \times 10^{-2}$ M sodium malonate as a competitive inhibitor.

The potential changes in the permeability after the administration of Novurit were attempted to demonstrate with a fluorescent microscopic method for extravasal protein using Thioflavin S and Euchrysin 2 GNX as fluorochromes (10), and with ninhydrin-Schiff staining for amino groups in proteins according to the method of Yasuma and Ichikawa (25). Delafield's haematoxylin-eosin staining was employed in order to detect any morphological changes discernible with routine methods.

#### RESULTS

*Control Animals.* — In the kidney sections of control rats treated according to the method of Barnett and Seligman for sulfhydryl groups (Fig. 1), the glomeruli showed a purplish colour suggesting sparse, widely separated -SH groups. The convoluted tubules, both proximal and distal, were blue indicating a great concentration of -SH groups. The intermediate zone between the cortex and medulla consisting principally of the straight terminal portions of the proximal convoluted tubules and portions of the Henle's loops was also blue of -SH groups as the convoluted tubules. Macroscopically the intermediate zone was even more intensely coloured than the cortex with the fainter stained glomeruli. The medullary area of Henle's loops was reddish-blue indicating a smaller concentration of -SH groups. The collecting ducts in the papilla appeared to be less intensely reactive showing only a reddish colour. The circulatory endothelium revealed a slight, the media a great concentration of -SH groups.

The unfixed frozen kidney sections of control animals treated with the ferric-ferricyanide reduction tests revealed in different shades of greenish-blue a similar distribution of the staining intensity

<sup>1</sup> Obtained from the Montclair Research Corp., Montclair, New Jersey.

<sup>2</sup> Obtained from Light & Co.



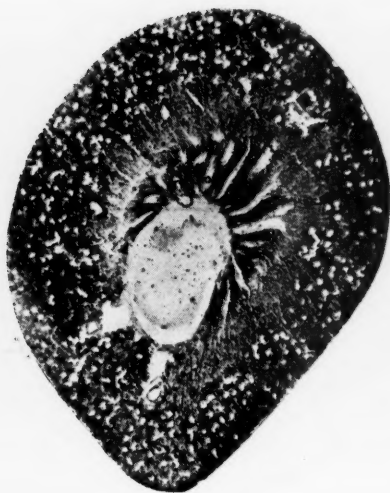


Fig.2

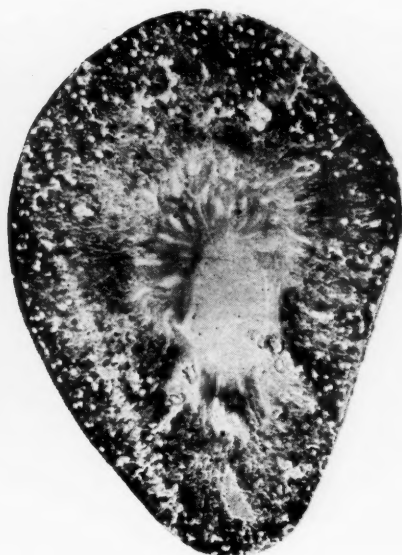


Fig. 3

Fig. 2. — The distribution of succinic dehydrogenase in normal rat kidney. The section of  $80\ \mu$  was treated according to the method of Seligman and Rutenburg employing BT as indicator. Note the sharp line against the unstained papilla where the loops of Henle bent to re-enter the kidney cortex.  $\times 7$ .  
Fig. 3. — Succinic dehydrogenase activity of rat kidney 3 hours after a subcutaneous administration of Novurit, 20 mg Hg/kg of body weight. Note the nearly complete lack of pigmentation in the medullary area of Henle's loops  
Section thickness  $80\ \mu$ .  $\times 7$ .

The collecting ducts were faintly reactive, if at all. The circulatory endothelium was always unstained.

In the kidneys of the control animals there were very small amounts of extravasal protein in some of the glomeruli and even around them, and in the papilla as revealed by the brownish-red secondary fluorescence. Protein casts in the tubuli were not found with the ninhydrin-Schiff staining.

*Test Animals.* — After administration of Novurit the distribution and staining intensity of protein-bound-SH groups remained unchanged in the nephrons despite the fact that the highest doses were generally lethal. The ferric-ferricyanide reduction methods and the nitroprusside tests also revealed no differences in the staining of the kidney sections as compared with the controls.

When BT was used as an indicator for succinic dehydrogenase

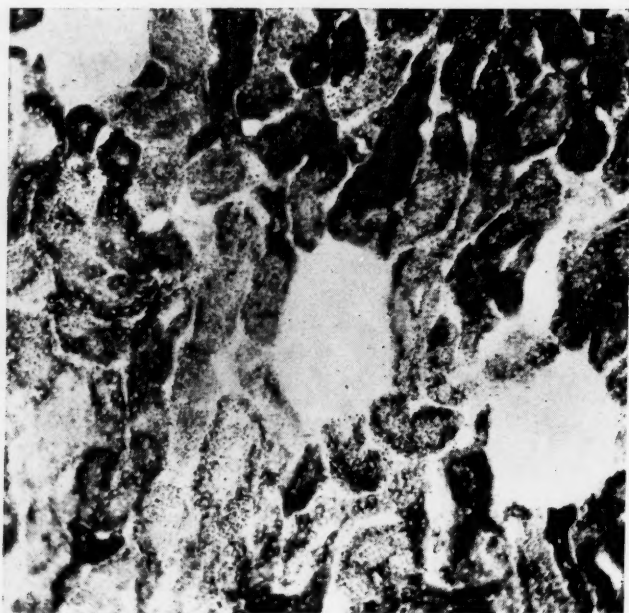


Fig. 4. — A BT preparation showing a normal succinic dehydrogenase activity in the cortex of rat kidney. Section thickness  $30\ \mu$ . Note the unstained glomeruli and nuclei. Especially the proximal convoluted tubules when bulging from the glomeruli are heavily deposited with fine particulate crystals of diformazan.  
 $\times 175$ .

activity in the kidneys of the test animals, a general decline was noticeable in the enzymatic activity. Especially the medullary portions of Henle's loops and the tubular segments radiating through the intermediate zone were inhibited in their succinic dehydrogenase activity. The activity was depressed less strongly in the cells of the convoluted tubules, both proximal and distal (Fig. 3). The degree of the depression in the enzymatic activity was directly proportional to the doses of Novurit. The doses of 10 mg Hg/kg caused a moderate or slight diminution in the formazan precipitation of Henle's loops, the pigmentation of the cortex remaining unchanged. The succinic dehydrogenase activity of the convoluted tubules was not clearly depressed before the doses were 20 mg Hg/kg or more (Fig. 5.). In sections of  $25\text{--}30\ \mu$  the doses of 40 mg Hg/kg caused a complete lack of staining except in some of the sections; in these the Henle's loops were unstained and a

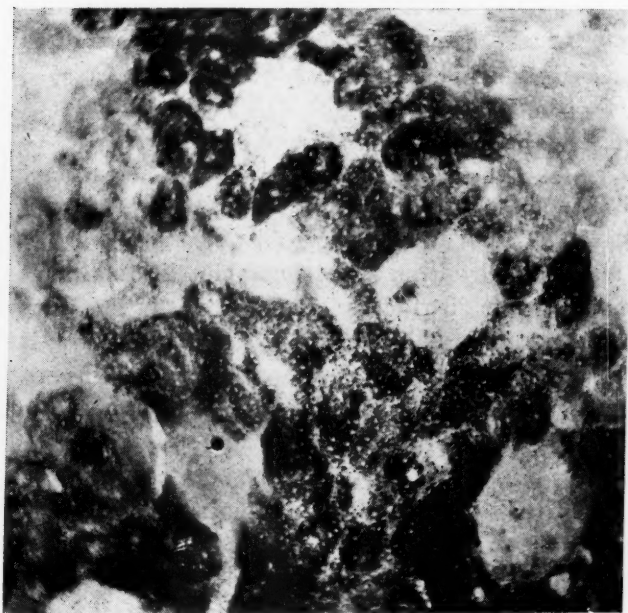


Fig. 5. — A BT preparation from kidney cortex of rat after administration of Novurit, 25 mg Hg/kg. Section thickness 30  $\mu$ . Note the decreased succinic dehydrogenase activity both in distal and proximal convoluted tubules and the irregular deposition of formazan crystals.  $\times 175$ .

reddish shade could be seen in the cortex. In sections of 50—100  $\mu$  a complete loss of pigmentation was noticeable only in the cells of Henle's loops.

Using NT or TPT as indicator for succinic dehydrogenase activity a general diminution in the deposition of formazan granules was also observed. However, such a complete lack of staining in the cells of Henle's loops as in the BT preparations could not be established, not even after the greatest doses of Novurit. The activity of Henle's loops was anyhow more depressed than that of the cortex.

In fluorochromic examination for extravasal protein there was a moderate increase in the glomeruli and their neighbourhood after the highest doses of Novurit. The doses over 25 mg Hg/kg called forth also protein casts in some of the tubuli demonstrable with the ninhydrin-Schiff staining.

After the highest doses of Novurit the routine haematoxylin-eosin staining revealed in some of the sections hyperemic areas and somewhat engorged tubular cells.<sup>1</sup>

#### COMMENTS

The demonstration of protein-bound-SH groups according to Barnett and Seligman resulted in all preparations in a clear-cut colour reaction with adequate histological localization. Our observations regarding the distribution of -SH groups in the kidney are in agreement with the results of Barnett (4), except that in all our preparations the cells of Henle's loops reacted slightly but clearly, whereas Barnett stated that the thin limbs were »virtually negative».

The poor histological localization and the rapidly fading colour rendered the nitroprusside methods unapplicable for the present investigation.

The ferric-ferricyanide reduction test as modified by Lillie and Burtner presented relatively clearly the histological structure, but the reagent is reduced to Prussian blue, besides -SH groups, by several compounds including ascorbic acid and uric acid (15). In addition, the pre-treatment of sections with saturated  $\text{HgCl}_2$  (18), did not result in a complete loss of staining, and these methods, therefore, were considered to be unsuited for the demonstration of mercurial action on -SH groups. Using the Chèvremont-Frédéric modification, Halmágyi, Kovács, and Varró (12), however, have described a fall in the concentration of -SH groups in kidneys of dogs after administration of Novurit, an observation which we could not confirm in the kidney of rat.

The specificity of the Seligman-Rutenburg-method for the demonstration of succinic dehydrogenase was ascertained by following procedures. When succinate was omitted from the incubation mixture no reaction occurred if frozen sections were used. On the contrary, fresh unfrozen kidney slices were clearly stained even without succinate indicating dehydrogenase activity acting on endogenous substrates. This activity was effectively destroyed by freezing. Shelton and Schneider (22) have assumed that freezing separates some components of the enzymatic reaction system of the

<sup>1</sup> We are indebted to Prof. Kai Setälä, M.D., for his valuable criticism.

cell, such as substrate and enzyme, thus breaking the chain of electron transfer.  $2.5 \times 10^{-2}$  M sodium malonate, the specific competitive inhibitor, caused also a complete lack of staining.

In sections treated with blue tetrazolium or neotetrazolium the fine blue or purple-red formazan granules, respectively, were distinctly intracellular, and in any cross section of a single tubule there was very little variation in the deposition of the small crystals whereas the deep-red reduction product of triphenyltetrazolium was frequently unequally precipitated as large, flaky and partly extracellular crystals of various size and shape which obscured the cellular details. When BT was employed the small size of diformazan crystals made it possible to discern even more easily some cellular structures, e.g. the unstained nuclei, than with NT. On the other hand, NT is more sensitive in the demonstration of succinic dehydrogenase than BT. It stains thinner sections more uniformly, reacts more rapidly, and it demonstrates succinic dehydrogenase activity in several tissues which remain unstained with BT. In anaerobic conditions (20), however, also the thinner BT-sections stained uniformly. The addition of ionic activators to the incubation medium was useless. This was also noticeable in sections of heart, an organ which likewise possesses a high succinic dehydrogenase activity (23).

Our observations regarding the distribution of succinic dehydrogenase in nephrons are in agreement with the results of Rutenburg, Wolman, and Seligman, and with those of Shelton and Schneider.

The complete absence of precipitated formazan in the cells of Henle's loops observed in the BT-preparations after administration of Novurit does not indicate a complete inhibition of succinic dehydrogenase activity as is revealed from the moderate pigmentation of these cells in the parallel sections treated with NT. Moreover, the sensitiveness of various tetrazolium salts in the determination of succinic dehydrogenase activity has, to our knowledge, hitherto not been compared with that of the earlier biochemical methods.

Theophylline, a constituent of Novurit, probably plays no rôle in the results obtained, since it in concentrations up to  $5 \times 10^{-3}$  M has no effect on the succinic dehydrogenase system (14).

In the toxicity determination, the doses over 40 mg Hg/kg appeared to be generally lethal within 3 days. In the animals who died, the death occurred with jerky movements. The changes in animals who survived were reversible in 3–5 days.

## DISCUSSION

Duggan and Pitts (9) assumed that a large dose of a diuretic could completely block reabsorption in that segment of tubule on which it acts. According to them, the proximal segment is responsible for twothirds or more of the total renal reabsorptive capacity for water and electrolytes. Their observation that large doses of mercurial diuretics block about 20 per cent of the renal reabsorptive capacity led them to suggest that mercurials act on the distal segment (9, 19). Our result that the main depression of succinic dehydrogenase activity after administration of mercurials takes place in the loops of Henle where the facultative reabsorption of water is believed to begin, supports this view (16).

The potential diminution of sulfhydryl groups in the kidney cells after administration of mercurial diuretics could not be demonstrated with the histochemical methods used (24). This result, however, is not an indication against the assumption that mercurial diuretics act by combining with the essential -SH groups of enzyme proteins, considering that the vast majority of the demonstrable -SH groups are not directly concerned with these enzymes. The observation that the most pronounced inhibition of succinic dehydrogenase activity occurred in Henle's loops of the medullary area where the concentration of -SH groups is relatively sparse, draws ones attention to the possibility that the proportion of the essential -SH groups of enzyme protein to the more or less indifferent -SH groups of cell is greater in the cells of Henle's loops than in the convoluted tubules. Consequently, the -SH groups of succinic dehydrogenase in the cells of Henle's loops seem to be more apt to the blockage by mercurial diuretics calling forth the marked inhibition of this enzyme in the corresponding segments of the nephrons.

## SUMMARY

The subcutaneous and intramuscular administration of Novurit to rats in doses of 10–50 mg Hg/kg of body weight called forth a depression of the histochemically demonstrable succinic dehydrogenase activity of kidney, especially in the cells of Henle's loops. No change in the concentration of sulfhydryl groups could be

discerned by the histochemical methods employed. The results are discussed in the light of the earlier presented evidence for the distal tubular action of mercurial diuretics.

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## THE CAPACITY OF SMALL AMOUNTS OF INFLUENZA VIRUS TO DESTROY EGG MUCIN INHIBITOR

by

KARI PENTTINEN<sup>1</sup> and VEIKKO TOMMILA

(Received for publication December 30, 1953)

Several mucoproteins or mucopolysaccharides are capable of inhibiting the red cell agglutination produced by inactivated influenza viruses (7, 8, 10, 18). An active virus again is capable of destroying the inhibitory property of mucin (1, 2, 10, 17). The reaction is considered enzymatic in character (2, 9, 12). A similar effect is produced e.g. by *V. cholerae* enzyme and trypsin (4, 16). With a virus enzyme reacting on mucin a hexosamine type component is split off and its inhibitor property disappears (10). The capacity of destroying mucins has been found to be connected with the virus particle (11). The different influenza viruses possess a mucinase activity of varying degree (2).

The inactivation of mucin is proportionate to the amount of virus. The destruction of the substratum with large amounts of virus occurs logarithmically as a function of time (2). Ion concentrations have been attributed a very great importance in the reaction (3). By contrast, opinions differ as to the necessity of calcium ions (3, 5, 14). Optimal mucin destruction has been found to occur at a temperature of +37°C and a pH of 6.2 (2). In the investigations referred to above large amounts of virus have been used, as a rule several or several tens of agglutinating units, and with a suitable amount of substratum the time required for the destruction of mucin is relatively short, at the most a few hours. In 1952, studying the capacity of different influenza viruses to destroy nasal mucin

<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

secretion Fazekas de St. Groth was able to destroy mucin with considerably smaller amounts of virus than the agglutinating unit, using a short reaction time (6).

In the present investigation we have studied the minimum quantities of virus (infl. A/Finland/1/51) that suffice to produce demonstrable destruction of egg mucin with a long reaction time of 24 hours and whether it is possible in this way to show in the test tube smaller amounts of virus than with hemagglutination. In the investigations effected we found that with virus-mucin reaction occurring in broth or albumin solution a demonstrable decomposition of mucin is achieved with much smaller amounts (1/16—1/100) of virus than in physiological saline solution, in which demonstrable destruction of mucin occurred at titres corresponding to the haemagglutinin titre.

#### THE PRESENT INVESTIGATION

*Virus.* — The virus employed in the investigation was infl. A/Finland/1/51/E<sub>27-30</sub>. The infected allantoic fluids (haemagglutination titres 1/4096) were used as fresh as possible.

*Indicator virus.* The strain employed was the Lee strain, of influenza B(F8 M139 E<sub>168-171</sub>). The infected allantoic fluid was inactivated by heating for 30 min. at +56°C.

*Mucin.* — The substratum employed consisted of inhibitor (13) purified from eggwhite with distilled water precipitation in cold and ultracentrifugation.

*Broth.* — The broth employed contained 3 g meat extract, 10 g peptone (Witte), 5 g NaCl in 1000 cc of distilled water and was sterilized in autoclave. pH corrected by NaOH and HCl to 7.2.

*Albumin Solution.* — The albumin solution contained 1% human albumin in buffered physiological saline solution, pH 7.2.

*Physiological Saline.* — The physiological saline solution employed was isotonic, 0.9%, buffered with 0.06 M phosphate buffer, pH, 7.2.

*The Inhibitory Activity of Mucin.* — It was titrated by diluting the mucin 1: 20 and preparing a twofold dilution series into buffered physiological saline, adding to 0.25 cc of the dilution 4 agglutinating units of indicator virus in 0.25 cc, letting the mixture stand 30 min. at room temperature and then adding 0.5 cc of 0.5% chicken cells.

Reading was taken after an hour. The last dilution to inhibit completely the hemagglutination caused by the virus was taken as the inhibition titre if the next tube showed complete agglutination, but if the agglutination was partial the titre was interpolated at the geometrical mean of the dilutions. Virus control showed that the indicator virus amount employed in the test was 4 agglutinating units. All titrations were effected on plastic plates (15).

The inhibition titre of mucin treated with virus in the experiments was determined in the same way, except that the dilution series was prepared direct from undiluted reaction mixture.

*The Capacity of small Amounts of Virus Infl.A/Finland/1/51 to Destroy Egg Mucin Inhibitor in Various Media.* — A twofold dilution series, starting from 1: 500, was prepared from infl.A/Finland/1/51/E<sub>27</sub> (aggl.titre 1/4096) (a) in broth, (b) in physiological saline solution, and (c) 1% human albumin solution. Purified egg inhibitor (inhibition titre 1/320) was diluted in identical solution (a, b, c) 1: 10, with 500 units/cc of penicillin and 500  $\gamma$ /cc of streptomycin added to prevent infection. 0.5 cc of inhibitor dilution and 0.5 cc of virus-allantic fluid dilution in the corresponding dilution fluid (a, b, c) was measured into test tubes. The tubes were sealed, shaken and placed in a water bath at +37°C for 24 hours, shaken from time to time. The control consisted of a tube with an identical inhibitor dilution in broth. Similar control tubes were also prepared from the albumin solution and physiological saline solution. The day after the inhibitory activity of the virus-treated mucin was titrated

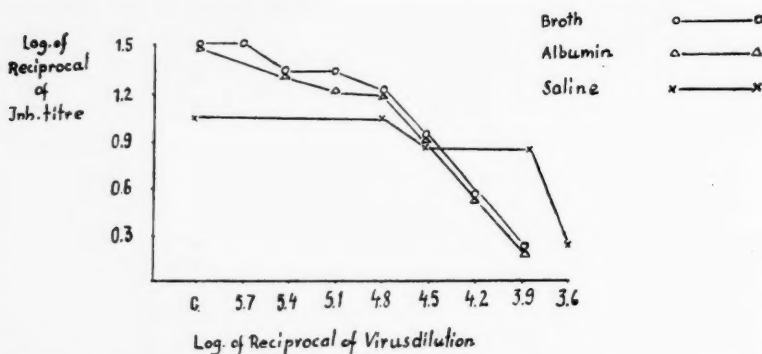


Fig. 1. — The capacity of small amounts of virus Infl.A/Finland/1/51 to destroy egg mucin inhibitor in various media. Reaction time 24 hours, temperature +37°C. The titres of inhibitor controls in various media are entered at the point C.

with indicator virus (the virus present in the tubes was not destroyed before titration as the virus amounts employed were generally smaller than the agglutinating unit and the dilution involved in titration, in addition, was 4-fold). The titres obtained were then compared with those of the control tubes entered in the table at the C points.

The curve shows, firstly, that the inhibitor is considerably more actively present in broth and albumin solution than in physiological saline solution. Secondly, if the reaction takes place in albumin solution a 1: 128,000 (5.1) virus dilution produces a drop of 0.3 log. in the titre of the inhibitor which corresponds to an approximately 50% destruction of inhibitor mucin. A similar destruction occurs in broth when virus is diluted 1: 64,000 (4.8). But a definitely demonstrable decomposition of the inhibitor in physiological saline solution is only produced by a 1: 4,000 (3.6) virus dilution, which with the virus concerned equals the haemagglutinin titre. With larger virus quantities the destruction of mucin is proportionate to the amount of virus. We repeated the experiment several times, getting results on the same lines although there seemed to be considerable differences between the various batches of broth and albumin. Infl. B (Lee) behaved similarly in our experiments. With a substratum of filtered, centrifuged eggwhite diluted 1: 100 the results were similar, although, in physiological saline solution, a drop of 0.3 log. in the titre was obtained with virus amounts smaller than the above. This result seems to comply with those obtained in the investigation of nasal mucin (6).

We tried to discover whether the capacity of small amounts of virus to decompose mucin inhibitor, greater in broth and albumin solution than in physiological saline solution, was due to the greater mucinase activity of the virus itself in those solutions or to the higher stability of virus properties in them during the prolonged incubation. To do this we determined the infection titre of the virus in broth and physiological saline solution before and after the incubation of the virus-mucin mixture, and the stability of the mucinase activity of the virus during incubation.

*Changes in the Infection Titre of the Virus during the Incubation of Virus-mucin Mixture.* — A serial dilution up to  $10^{-11}$  was prepared from infl. A/Finland/1/51/E<sub>27</sub> (haemaggl. titre

1/4096) both in broth and physiological saline solution. An addition of 1/10 of inhibitor (inhibition titre 1/640) diluted in broth was made to the virus dilutions in broth (0.5 cc) and, similarly, inhibitor diluted in physiological saline was added to virus dilutions in physiological saline (0.5 cc). Immediately after the addition and shaking 0.2 cc of each virus dilution was inoculated into the allantoic sacs of five eggs incubated for 11 days. A similar procedure was repeated with the same mixtures after incubation for 24 hours at  $+37^{\circ}\text{C}$ . After the eggs had been in the incubator ( $+36^{\circ}\text{C}$ ) for 48 hours the allantoic fluids were collected and their agglutinating ability was studied. 60% positivity was selected as the infection titre. Similarly a control infection titre was determined on virus in pure broth and after the virus dilutions had been in a  $+37^{\circ}\text{C}$  water bath for 24 hours. The inhibition titres of the mixtures were determined as described above.

Fig. 2 shows the inter-connection between the mucinase activity and infection titre of A/F virus in broth and physiological saline.

In the broth a drop of 0.3 log. in the inhibition titre of the substratum was obtained in the experiment with virus dilution  $10^{-5}$  (5). At the same time the infection titre of the virus dropped from  $10^{-7}$  (7) to  $10^{-5}$  (5), i.e. by a hundredth. An equal drop in infection titre occurred in the control test effected in pure broth, even

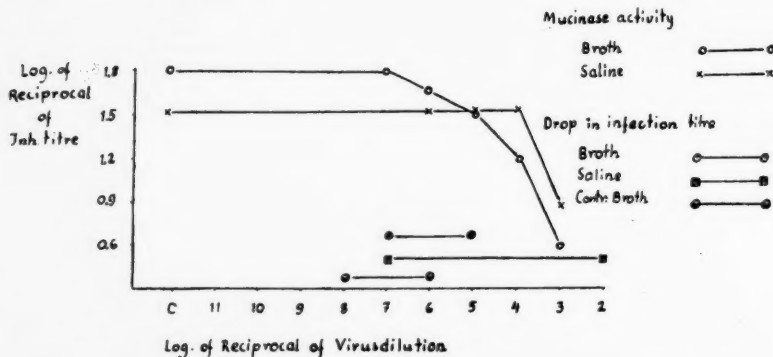


Fig. 2. — The capacity of minute amounts of virus infl.A/Finland/1/51 to destroy mucin in broth and physiological saline. Reaction time 24 hours, temperature  $+37^{\circ}\text{C}$ . During the experiment the infection titre of the virus in inhibitor-containing broth dropped from  $10^{-7}$  to  $10^{-5}$  and in physiological saline from  $10^{-7}$  to  $10^{-2}$ . In the control broth the titre dropped from  $10^{-8}$  to  $10^{-6}$ .

though the titres were ten times higher. In a test made in buffered physiological saline solution a 50% destruction of the inhibitor was only obtained with a dose 100 times larger, i.e.  $10^{-3}$  (3), than in broth. At the same time the infection titre dropped from  $10^{-7}$  to  $10^{-2}$  (2), i.e. by a 100,000th. In buffered physiological saline, therefore, the drop in infection titre was 1,000 times greater than in broth.

*Changes in the Mucinas Activity of the Virus in Broth and Physiological Saline within 24 Hours of Incubation at +37°C.*—

In the previous experiment the long incubation period induced a much greater drop of infection titre in physiological saline than in broth, as was to be expected. The behaviour of the mucinase activity of virus during a corresponding period under similar conditions was unpredictable. Burnet had found that one and the same amount of virus was capable of destroying one amount of mucin after another but the reaction periods in the experiments were short and the virus amounts large (2). In order to study the possible changes in mucinase activity of virus during the incubation period the mucinase activity of the virus studied was determined before and after 24 hours' incubation at +37°C both in broth and in physiological saline solution.

A serial dilution  $10^{-3}$ — $10^{-6}$  was prepared from infl. A/Finland/1/51/E<sub>30</sub> (haemaggl. titre 1/4096) both in broth and buffered physiological saline solution. 1:10 dilutions of the inhibitor (inh. titre 1/640) were made in broth and physiological saline solution. The virus dilutions contained the same amounts of penicillin and strepto-

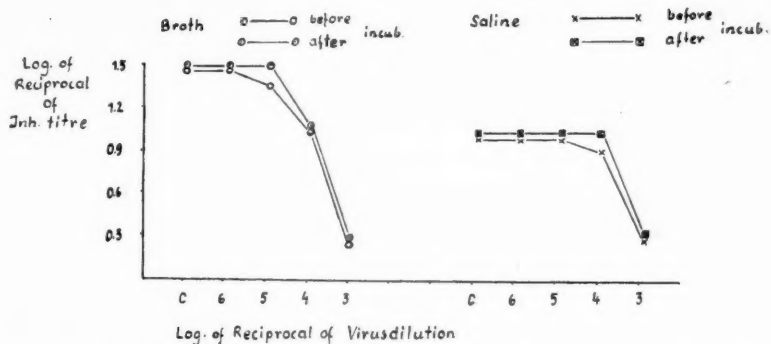


Fig. 3. — Changes in the mucinase activity of virus infl. A/Finland/1/51 during 24 hours' incubation at +37°C

mycin as above. The mucinase activity of the virus dilutions was determined by combining 0.5 cc of virus dilution and 0.5 cc of inhibitor dilution and by incubating the mixture for 24 hours at  $+37^{\circ}\text{C}$ . Upon this the remaining inhibition activity was titrated and control values were determined simultaneously. Then the mucinase activity of the virus dilutions was re-determined after they had been kept for 24 hours at  $+37^{\circ}\text{C}$ .

A review of the result shows that in this experiment too the virus had a better capacity for destroying mucin in broth than in physiological saline solution. In addition it is evident that in both media 24-hour incubation at  $+37^{\circ}\text{C}$  reduces, the mucinase activity of the virus to a limited extent only. The same was found also in experiments in which new mucin was added to a virus-mucin mixture incubated for 24 hours at  $+37^{\circ}\text{C}$ , in which the substratum had undergone decomposition. The mucin added was decomposed by identical virus quantities approximately to the same extent as the original mucin.

#### DISCUSSION

The investigations seem to show that albumin solution and broth are more favourable media for virus-mucin reaction than buffered physiological saline solution. Virus amounts 16—100 times smaller than required with physiological saline induce a demonstrable mucin destruction in the first two on substratum containing purified egg mucin inhibitor. The readier decomposition of mucin in broth than in physiological saline solution, according to our investigations, is not due to a virus-protecting influence of factors present in broth during the long incubation. The infectivity of virus is preserved much better in broth than in physiological saline but the weakening of its mucinase activity is similar in the two media and very slight. The greater mucinase activity of virus in broth is probably due to thus far unknown factors in the broth. The results in albumin medium corresponded to those obtained in broth.

The different behaviour of purified and native inhibitor is perhaps accounted for by the assumption that the latter, in addition to the inhibitory component, contains other components (possibly similar to those of broth or albumin) in larger quantities than the purified inhibitor employed in the experiment proper.

The investigations also reveal the distinct difference between the infectivity and mucinase activity of virus. This emerges particularly well in physiological saline solution in which the drop in infectivity during incubation is 100,000-fold against a barely-observable drop in the mucin-decomposing activity.

A demonstrable destruction of mucin in broth and 1% albumin solution was achieved with virus titres equalling 1/16—1/32 of the haemagglutinin titre. In some experiments a demonstrable decomposition of mucin could be achieved with even smaller amounts of virus. A more favourable result still might be possible if optimal conditions were found for the virus-mucin reaction. Investigations into this point continue. A study of the practical significance of the phenomenon, the demonstration of influenza virus in throat washings, taking into account also the adsorption into and elution from red cells and the use of anti-sera, is under way. In any case it can be said that in the above conditions it is possible to demonstrate the presence of smaller amounts of virus with the capacity of influenza virus to destroy mucin inhibitor than with red cell agglutination.

#### SUMMARY

The investigation dealt with the capacity of minute amounts of infl.A/Finland/1/51 virus to destroy mucin inhibitor. It was found that by effecting the virus-mucin reaction in broth or in albumin solution and by using purified egg inhibitor as the substratum, demonstrable destruction of mucin is achieved by virus amounts 1/16—1/100 smaller than if the reaction were induced in buffered physiological saline solution, in which demonstrable mucin destruction was obtained by virus titres equalling haemagglutination. During the 24-hour incubation period employed for the reaction the drop in the infection titre of the virus was in saline 1,000 times bigger than in broth where the drop was approx. 100-fold. During the same incubation period the drop in the mucinase activity of virus both in broth and in physiological saline was slight only. The greater mucinase activity of virus in broth and albumin solution, on the basis of the experiments effected, is a result of the more favourable conditions for the reaction in these media and not of the higher stability of the virus in them during incubation.

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TRANSPLANTATION OF HUMAN NORMAL AND NEOPLASTIC TISSUE TO THE CHORIO ALLANTOIC MEMBRANE OF EMBRYONATED CHICKEN EGGS<sup>1, 2</sup>

by

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(Received for publication January 8, 1954)

The experiments presented in this paper were initiated some three years ago to continue a previous work concerned with the behaviour of chicken and duck tumors in the embryonated eggs of chickens and ducks (8). The purpose was partly to investigate the ability of human tumors to grow on the chorio allantoic membrane (CAM) of the embryonated egg, partly to find out if such growths would induce lesions in the embryo similar either to the hemorrhagic disease following injection of the Rous sarcoma virus into the embryo as described by Milford and Duran-Reynals (6), or oedema following grafts of Hodgkins disease tissue to the CAM as reported by Karnowsky and coworkers (4). In other words, to ascertain whether the embryo of embryonated chicken eggs could serve as an indicator of some hypothetical tumor-producing factor occurring in human tumors.

Goodpasture, Douglas and Anderson have reported on successful grafts of human skin, moles and keloids to the CAM of embryonated chicken eggs (2) which findings have been confirmed by Blank, Coriell and McNair Scott (10), but attempts to transplant

<sup>1</sup> This investigation was aided by grants from Sigrid Juselius' Stiftelse.

<sup>2</sup> A preliminary report on the results presented in this paper was given at a meeting of Finska Läkare-Sällskapet on the 27th of November, 1952.

<sup>3</sup> We wish to express our sincere thanks to Professor Ivar Wallgren, MD, who has kindly offered his criticism and valuable help in interpreting the microscopical preparations.

human malignant tissue to the CAM had, when this work was started, with few exceptions been less successful (3, 4, 9). For this reason and because, as was apparent from the introductory experiments, the interpretation of the results, as far as the reaction of the CAM to the transplants was concerned, was somewhat difficult, the work was mainly concerned with the transplantability of human tumor tissue, and as controls human embryonic and adult tissue, to the CAM of embryonated chicken eggs. During this time, however, two more extensive reports on the use of the embryonated chicken egg for the transplantation of both animal and human tumors have been published. The publication of Karnowsky and coworkers (5), also gave a brief survey of the literature in this field beginning with the original work of Murphy (7). As far as human tumors are concerned their report is based on transplantation experiments with tissue from a total of 71 human tumors including tissue from Hodgkins disease, lymphosarcomas, lymphatic leukemia, carcinoma of the lung, mammary carcinomas and melanomas, and the conclusion reached concerning this part of the work is as follows: «The transplantation of human carcinomas has been sporadically successful. For example, if ten eggs are implanted with fragments of carcinoma tissue three may sometimes show survival of the tumor on the CAM by an enlarged nodule at the site of implantation and the presence of human tumor cells on microscopic examination, although in no instance could these tumors be transplanted successfully to the CAM of other eggs.» The other report, published by Sheldon, Sullivan and Warren (11) concerns 59 different human cancers transplanted on the CAM. These investigators stated that sarcomas survived better on CAM than carcinomas.

The results of our study confirm partly the above given statements. As far as the human skin and moles are concerned the results obtained have mainly been similar to those reported by Good-pasteur, Douglas and Anderson. Our experiments are, however, mainly concerned with the transplantation of carcinomas of the human skin and especial stress has been laid upon the transplantability of these grafts to the CAM of other eggs and upon the reaction of the membrane to these grafts. We therefore feel that the presentation of these results, although by no means conclusive, may add to the knowledge of the behaviour of human tumor tissue on the chorio allantoic membrane of embryonated chicken eggs.

## MATERIAL AND METHODS

## CONTROLS

*Normal Homologous Tissue.* — Skin from 10-day old chicken embryos; chorio allantoic membrane from 18-day old embryonated eggs.

*Normal Heterologous Tissue.* — Skin from human embryo (three months of age); skin from human adults; human placenta tissue at different stages of development.

*Other Controls.* — With the intention of following the reaction of the CAM to nonspecific stimuli physiological saline containing penicillin and streptomycin was dropped onto the membrane. As another control sterile sand was explanted to the CAM.

## TUMORS

Tissue of the following 39 human tumors were transplanted to the CAM: Naevus (4), Melanoma (2), Carcinoma spinocellulare (11), Carcinoma basocellulare (2), Carcinoma labi (4), Carcinoma mammae (9), Carcinoma penis (1), Carcinoma ventriculi (3), Carcinoma uteri (1), Carcinoma pulmonum (1), Fibrosarcoma (1), Sarcoma tibiae (1), Papilloma of the larynx (3).

## METHOD OF EXPLANTATION

*Tissue Fragments.* — All the tissue was freshly obtained by biopsy and transplanted within two hours of removal from the source. The tissue was cut into pieces of about 1 mm in diameter and soaked in sterile saline containing 50 units of penicillin and 50 micrograms of streptomycin per ml. Embryonated eggs 7 to 11 (usually 9 to 10) days of age were used. A window was cut in the shell over the vascular area, the membrane was dropped and a piece of the tissue was placed on or close to a big blood vessel. The window was sealed with scotch tape and the eggs were incubated at 37° C for from 2 to 13 days. In some cases the embryos were allowed to hatch in order to follow the reaction of the chickens obtained from eggs inoculated with the different material.

*Cell Suspension.* — The tissue was minced with scissors and ground up in a mortar. Some saline-containing penicillin and

streptomycin were added as above. Of the cell suspension 0.1 ml was dropped on to the CAM and the eggs incubated as above.

*Tissue Extract.* — The cell suspension was centrifuged at 2500 rpm for 15 minutes and the supernatant fluid inoculated and the eggs incubated as above.

#### TRANSPLANTATION TO THE CAM OF OTHER EGGS

The possible growth on the CAM was cut into two or several pieces depending on the size of the growth. One of the pieces was taken for sectioning and the others explanted on to new membranes and incubated as above.

#### HISTOLOGICAL EXAMINATION

Tissue was fixed in ten per cent formalin, cut at ten microns and stained with hematoxylin and van Giesons stain.

#### RESULTS

From the point of view of following the reaction of the CAM to the explanted tissue, it was, as stated in the introduction, of interest to follow the reaction of the membrane also to nonspecific stimuli. For this reason the membrane of several eggs was only dropped, the window in the shell sealed and the eggs incubated as in all the other experiments; these in all other respects normal membranes were sectioned after different times of incubation. In most of these membranes there were no lesions of the ecto- or endoderm. In some cases, however, slight papilliform proliferation of both the ecto- and endoderm could be observed, and the membrane was slightly thickened. Figure 1 shows one such membrane dropped at ten days of incubation and subsequently following incubation for eight days. Epithelial pearls can only very rarely be observed in such membranes. The reaction of the CAM to saline-containing penicillin and streptomycin is of the same degree as that shown in Figure 1. If sterile sand is dropped onto the CAM the result is a more marked proliferation of the mesoderm surrounding the foreign bodies, and foreign body giant cells may sometimes be seen. A moderate proliferation of the endo- and ectoderm also follows. In these cases epithelial pearls are now and then seen.



Fig. 1. — Chorio allantoic membrane to which nothing has been explanted. The membrane is thickened and shows oedema and proliferation of the mesoderm. At the arrow is seen the papilliform proliferation of the ectoderm mentioned in the text.

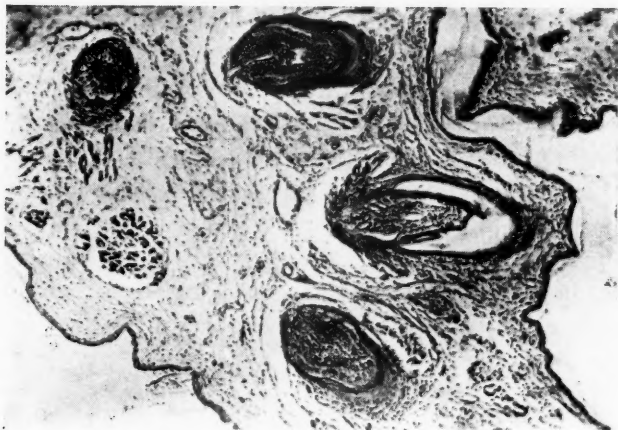


Fig. 2. — Chicken embryo skin explanted to the CAM. Incubated for 8 days. In the thickened membrane are seen four down follicles of the implanted chicken skin.



Figl 3. — Human embryonic skin explanted to the CAM. Incubated for 9 days. At the arrows are seen islands of epithelial cells which are of different appearance from the epithelial cells of the membrane. They apparently represent explanted human skin showing keratinization.

#### NORMAL HOMOLOGOUS TISSUE

*Chorio Allantoic Membrane.* — No growth of the inoculated membrane tissue could be observed and the CAM onto which it was explanted showed the same slight proliferation of the epithelium and mesoderm as the other controls.

*Chicken Embryonic Skin.* — Skin from 10-day old chicken embryos was explanted. Macroscopically there was good growth of the explanted tissue and even downs were seen growing in the implants. In many cases the implant was surrounded by a transparent sac containing clear fluid and in many respects similar to the CAM. A microscopical picture of the transplanted chicken skin is shown in Fig. 2. In a second passage some surviving tissue was still seen, but the third passage was completely unsuccessful.

#### NORMAL HETEROLOGOUS TISSUE

*Human Embryonic Skin.* — The experiments performed with human embryonic skin were very few and mostly unsuccessful. In some cases, however, the inoculated skin did apparently survive on the CAM. (Fig. 3.) But in most cases only a necrotic area overlying the membrane was seen, apparently the inoculated tissue. At this



Fig. 4. — Human full-term placenta tissue explanted to the CAM. Incubated for 9 days. In the center of the membrane are seen numerous papilliform bodies which are built up by a network of fine fibres of connective tissue. They are surrounded by cells reminiscent of trophoblasts (at the arrow).

point the membrane epithelium showed slight proliferation and oedema and proliferation of the mesodermal tissue was also seen.

*Human Adult Skin.* — In no case did the transplant of human adult skin succeed any better than the transplant of the embryonic skin. Sometimes possible takes were seen in form of surviving epithelial cells, but the cells stained poorly and did not appear to be in good condition, and transplantation to the CAM of other eggs always resulted in necrosis of the transplants.

*Human Placental Tissue.* — Several experiments were performed with human placenta tissue obtained from human placentas at different stages of development. Macroscopically the transplantation resulted in nodules from 1 to 10 mm in diameter. Microscopically the picture was very typical (Fig. 4). The endo- and ectoderm of the CAM did not seem to be affected and epithelial pearls were only rarely seen. In the mesoderm a well-defined area of well vascularized papillary bodies which were built up by a fine network of connective tissue were seen. The papillae were surrounded by one layer of giant cells containing one to several nuclei, very much of the same appearance as trophoblasts. Although these cells were also reminiscent of foreign body giant cells we are inclined to believe

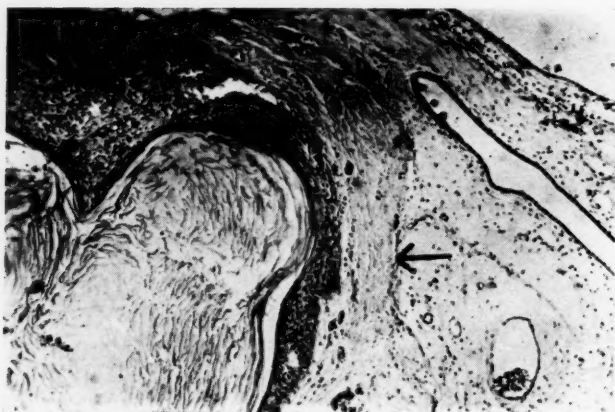


Fig. 5. — Naevus tissue explanted to the CAM. Incubated for 9 days. In the left corner is seen the surviving human epithelium showing keratinization. Between the connective tissue of the fragment and the mesoderm of the CAM there is a demarkation line (Arrow). No naevus cells can be observed.

that they really represented surviving or probably growing placenta tissue. The same picture was obtained by one or two passages on the CAM but further passages resulted in necrosis of the transplanted material.

#### TUMORS

Many of the tumors apparently did not grow or survive on the CAM. Entirely negative results were obtained with the mammary carcinomas, the fibrosarcoma, the carcinoma ventriculi, the carcinoma uteri, the papillomas of the larynx, the Carcinoma penis and sarcoma pulmonum. Macroscopically nodules of two to three mm in diameter were seen at the CAM around the explanted tissue fragment. The histological examination showed oedema and mesenchymal proliferation of the same type as in the controls, necrotic areas (probably inoculated tumor tissue) and sometimes slight proliferation of the ectodermal epithelium. As a rule no or very few epithelial pearls were seen.

*Naevus Pigmentosus.* — Fragments from 4 different naevi were transplanted to a total of 50 eggs. In several cases the fragments survived on the CAM for up to nine days (Fig. 5). The explanted



Fig. 6. — Melanoma tissue explanted to the CAM. Incubated for 8 days. The connective tissue of the fragment (see the arrow) contains chicken red cells and seems to fuse with the membrane tissue. The epithelium, which is covered by keratin, is in good condition, but no melanoma cells can be seen.

fragment was well vascularized. The cells and especially the epithelial cells appeared in fairly good condition. No naevus cells, however, could be detected. Transplantation to the CAM of other eggs resulted in all cases in necrosis of the transplanted tissue.

*Melanoma.* — Fragments of the melanoma were explanted to 37 eggs in all. The macroscopical and microscopical picture of the implants was much the same as that of the naevus fragments. No melanoma cells were seen and it is therefore most probable that the fragments explanted in this experiment actually consisted of more or less healthy skin (Fig. 6). For further transplantation nine eggs were used. The result was in all cases necrosis of the transplant.

*Carcinoma Basocellulare.* — The basocellular carcinomas were transplanted to 40 eggs in all. In some cases signs of survival and growth of the implanted tissue were seen (Fig. 7). The epithelial cells in the growths resemble those in normal human skin with epithelial downgrowths and the mesoderm of the membrane has small islands of small basal epithelial cells which are probably tumor cells. The ectoderm of the CAM often shows a marked proliferation with large vacuolized cells. A similar papilliform proli-



Fig. 7. — Basocellular carcinoma explanted to the CAM. Incubated for 8 days. In the left corner is seen surviving human epithelium covered by keratin. The cells resemble more those of healthy human epithelium than cancer cells. At the arrows, however, are islands of small basal epithelial cells which are probably cancer cells.

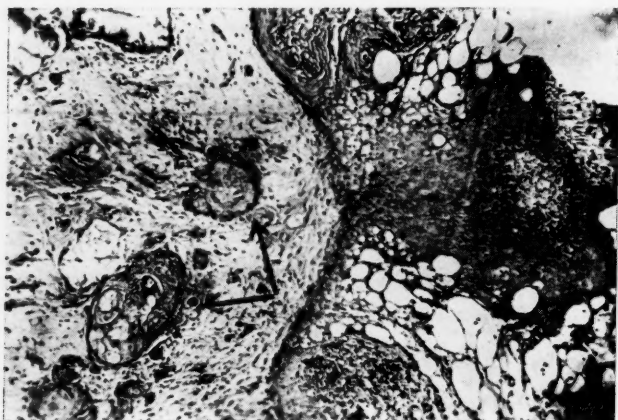


Fig. 8. — Spinocellular carcinoma explanted to the CAM. Incubation for 8 days. The cancer tissue has replaced the ectoderm of the CAM and no clear demarcation line can be seen between the cancer tissue and normal epithelium. In the mesoderm at the arrows are seen numerous islands of cancer cells surrounded by slight mesenchymal proliferation.



Fig. 9. — Spinocellular carcinoma explanted to the CAM. Incubated for 8 days. No surviving cancer cells can be seen in the picture, but at the arrows the marked proliferation of the ecto- and endoderm mentioned in the text can be seen. There is oedema and proliferation also in the mesoderm.

feration also sometimes occurs in the endoderm. Typical epithelial pearls may be seen.

*Carcinoma Spinocellulare.* — Fragments of different spinocellular carcinomas were explanted to the CAM of a total of 227 eggs. In no case could survival of the whole explanted fragment be detected, any more than with the naevus and the melanoma fragments. In some cases, however, explanted tumor tissue had apparently survived on the membrane and groups of epithelial cells resembling those in the original tumor were seen in the mesoderm surrounded by some mesenchymal proliferation. (Figure 8). In practically all the experiments the ecto- and endoderm of the CAM were markedly proliferated showing the same type of large vacuolized cells as those described in connection with the basocellular carcinoma experiments (fig. 9).

Attempts to transplant the possible growths to the CAM of other eggs were made in several cases. These second passages were mostly entirely unsuccessful in that the CAM showed only mesenchymal proliferation and necrotic areas and in some cases slight proliferation of the endo- and ectoderm of the CAM. In some cases, however, the ecto- and endoderm of the CAM were again markedly proliferated and the mesenchym was filled with numerous island

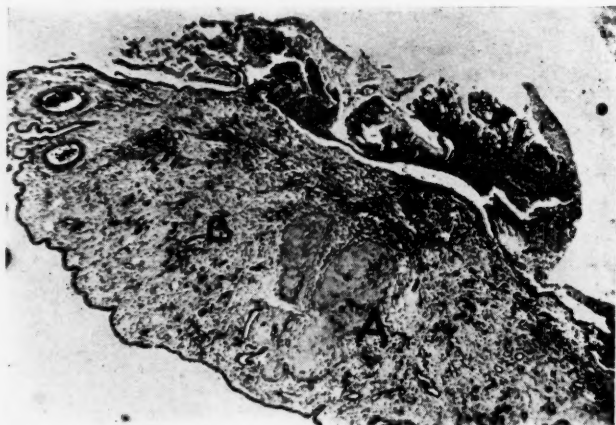


Fig. 10. — The picture shows a second passage of the spinocellular carcinoma. On the membrane is partly necrotic epithelium the nature of which can not be determined. In the membrane at A are islands of necrotic tissue, and at B numerous islands of cancer cells surrounded by slight mesenchymal proliferation.

of epithelial cells resembling tumor cells (Fig. 10). Further transplantation to a third passage only resulted in necrotic areas on the membrane and no typical lesions were seen in these membranes.

Cell suspensions of spinocellular carcinomas induced the same type of proliferation of the mesoderm and epithelium as did the fragments, but the explanted cells underwent necrosis and no growth could be observed. Transplantation experiments were unsuccessful.

Tumor extracts caused no typical changes in the CAM.

#### REACTION OF THE EMBRYO

As was mentioned in the introduction, no closer study of the embryos themselves were undertaken. All the embryos inoculated, however, survived without showing any macroscopical lesions of significance except in the comparatively few cases where contamination with bacteria or fungi occurred. In uncontaminated eggs oedematous embryos were sporadically seen as well as embryos showing roughened skin, but similar embryos were also seen among the controls and among uninoculated embryos.

## DISCUSSION

As was stated in the introduction, the purpose of this work, initially was to find out the reaction of the embryo of embryonated chicken eggs to human tumors growing on the chorio allantoic membrane. Because of the slight growth of the tumors, however, the main interest was concentrated on the behaviour of the explanted tissue on the CAM and on the reaction of the membrane to these explants compared with the reaction of the CAM to normal tissue and to non-specific stimuli.

We are fully aware of the fact that the experiments presented here are far from extensive enough to permit conclusions. Some of the results may however, have a certain interest for those who are concerned with the behaviour of malignant and non-malignant tissue on the CAM of embryonated chicken eggs.

As far as the reaction of the CAM to different stimuli is concerned, it can be stated that slight proliferation of the epithelium and proliferation and oedema of the membrane must be regarded as a normal reaction of the membrane independent of the stimuli used. Thus papilliform proliferation of the endoderm is very common, and epithelial pearls are seen now and then. This type of reaction is especially marked if corpuscular material like sand particles is placed on the membrane. Any foreign material, unless it is actually growing on the CAM, may thus in our opinion give rise to such foreign body reactions. Thus no conclusions can be drawn from this type of reaction e.g. as regards inoculated possibly proliferating agents. Although this fact has been pointed out earlier by others (1) we still feel it deserves especial consideration.

The growth of homologous tissue such as chicken embryo skin is, as was expected from earlier experiments, very good, and the results, including the transparent sac occurring around the explanted tissue, conform with previous conceptions. Although the fragments showed real growth on the CAM it does not seem possible to pass these grafts indefinitely.

The fact that Goodpasture, Douglas and Anderson (2), and Blank, Coriell, and Mc Nair Scott (10) have succeeded in transplanting human skin even through a second, sometimes fourth passage on the CAM shows that our technic in this respect has not been satisfactory. In very few cases only did our implants of human

embryonic and adult skin survive on the membrane. The reason apparently is that in these experiments the skin, contrary to the method of Goodpasture and coworkers, was cut into very small pieces; it therefore seems obvious that in most cases the epidermal surface and not the corium was brought into contact with the CAM, thus preventing the implants from being nourished from the membrane. In the case of the successfully transplanted naevus tissue, however, the fragments have been placed with the corium on the membrane. The results obtained with the naevus tissue also concur well with the results of Goodpasture and coworkers, although no successful second passages were attained. Similar positive results were obtained also in the experiments with the melanoma tissue where apparently healthy skin was explanted to the CAM.

Contrary to the skin which grew on the epithelium of the membrane, the placenta tissue always grew in the mesoderm and most of the inoculated membranes showed such growths. The explanted pieces of placenta tissue had apparently sunk down into the membrane where the growth then occurred, or where surviving placenta tissue could be seen.

Most of the tumors did not, as mentioned before, show any signs of growth or of survival of the tumor cells, and the results are thus in conformity with previous experiences. With the tumors of the human skin, however, some positive results were obtained. In the experiments with both the spinocellular and basocellular carcinomas there was undoubtedly survival of the explanted tissue, and in some cases this tissue showed something similar to infiltrative growth. Down-growths of the epithelial cells and islands of such cells were seen in the membranes. Although these cells appeared to be in fairly good condition mitoses were very seldom seen, and the tumors did not seem to be growing very actively. In most cases the second passage of such tumors were, as already stated, unsuccessful, but in some cases of the spinocellular carcinoma numerous islands of epithelial cells surrounded by slight mesenchymal proliferation were seen in the second passage. These cells, however, were difficult to distinguish from normal cells of the human epithelium and the possibility that they only represented epithelial pearls originating from the ectoderm of the CAM has of course also to be kept in mind. No such amount of epithelial pearls has, however, been observed in the other experiments and the epithelial pearls seen have been

of an entirely different appearance. We therefore feel that they really represent a second passage of the explanted tumor cells. In one case these cells seemed to be growing more actively than in the first passage and an adaption of the human carcinoma cells to the CAM could therefore be presumed perhaps. The fact that second passages of human tumors on the CAM have not been obtained earlier and the difficulty of interpreting the results, as well as the very few possibly positive passages, makes it necessary to assess the results critically and the experiments have of course to be repeated. In the case of tumors of the human skin it may, however, be possible to adapt the tumor cells to the CAM of the embryonated chicken egg.

Of some interest also is the very marked proliferation of the ecto- and endoderm of the membrane epithelium which appeared in most of the cases where tumor tissue of the human skin was explanted to the CAM. This same type of proliferation was also seen in some second passages and in the experiments where cell suspension of basocellular and spinocellular carcinomas was dropped onto the CAM. From the results obtained with the controls it was concluded that a slight proliferation of the membrane epithelium must be regarded as a normal reaction of the CAM. In no experiments, however, was such a marked proliferation of the epithelium seen. This may therefore be the result of some factor stimulating the epithelium to proliferation. Such proliferations could not, however, be obtained by using extracts of the tumor tissue and the cause therefore, without further experiments, still remains obscure.

As was already mentioned in the beginning of this discussion, we are fully aware of the difficulties of drawing conclusions from these results. The fact that the human skin and tumors of the skin seem to survive in connection with the epithelium of the CAM and the placenta tissue in the mesoderm makes it tempting to assume that the outcome of heterologous transplantation into the embryonated chicken egg is dependent on, among other factors, the type of tissue to which the cells are transplanted. Thus it could be assumed for instance that cells of a carcinoma of the liver would have a better chance of growing in the liver of the embryo than for example on the CAM. Although it is difficult to estimate the usefulness of the embryonated chicken egg for cancer research it seems that every possibility has not yet been explored and that the

embryonated chicken egg may thus aid studies concerned with the nature and properties of human tumors, especially if some human tumors could be adapted to the embryonated egg.

#### SUMMARY

The following human tumors have been explanted to the CAM of embryonated chicken eggs: Naevus pigmentosus, Carcinoma spinocellulare, carcinoma basocellulare, Melanoma, Carcinoma mammae, Carcinoma ventriculi, Carcinoma uteri, Carcinoma penis, Carcinoma pulmonum, Fibrosarcoma, Sarcoma tibiae and Papilloma of the larynx. As controls tissue of chicken embryo skin, human embryonic and adult skin and human placenta have been explanted in the same manner. As further controls sterile saline and sterile sand have been dropped onto the CAM.

Survival of the explanted tissue was seen to be sporadic with both heterologous and homologous skin and, very often, with human placenta.

In most of the experiments with tumor tissue the explanted tissue survive poorly on the CAM. Survival of explanted cells was seen in some cases with naevus tissue, Carcinoma basocellulare and Carcinoma spinocellulare, but it was difficult to determine whether any active growth occurred. Second passages were in most cases completely unsuccessful, but in a few cases possible second passages of spinocellular carcinomas were obtained.

Explantation of the skin carcinomas often resulted in very marked proliferation of the membrane epithelium in the environment of the implant. Similar proliferation but much less pronounced was also obtained if for example sterile saline or sand was dropped on the membrane. For this reason the interpretation of the reaction of the CAM to the implants seems to be very difficult.

If not contaminated by bacteria the embryos remained alive, but no microscopical examination was made of the embryos. In some cases oedematous embryos and embryos showing roughened skin were seen, but similarly affected embryos also occurred among the controls.

The reliability of the possible second tumor passages has been discussed, as well as the reaction of the membrane to the grafts. To be able to draw any conclusions the experiments must be re-

peated, but it is suggested that the embryonated chicken egg may be useful as a tool for further studies of the properties and nature of human tumors.

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## AETHER ANAESTHESIA

### INFLUENCE ON THE FORMATION OF INTRAVASCULAR RED CELL AGGREGATION IN CHILDREN

by

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(Received for publication January 12, 1954)

In the last thirty years Knisely, Bloch, Eliot and Warner (6) have developed a method by which the blood stream and red cells of the small blood vessels can be studied under the microscope in the conjunctival vessels. They have shown that the red cells of healthy men and animals are separate from one another and pass in single or double unbroken file through the capillaries. In larger vessels blood flows in a homogeneous mass, so fast that individual red cells cannot be distinguished. The red cells do not adhere to one another or to the vessel walls. They have also shown that in many conditions differing from the normal, e.g. in infectious diseases, pregnancy, delivery etc. and after a trauma the red cells circulating in the blood adhere to one another forming red cell clumps varying in size and viscosity depending on the strength of the factor producing aggregation.

Applying Knisely's method to children the present writer found in previous investigations (1) that no red cell aggregation occurred in the blood flowing in the conjunctival vessels of the newborn after a normal delivery, the red cells circulating singly without adhering to one another. After a delivery that was in some way abnormal, e.g. caesarean section or forceps delivery, or where the infant had large haemorrhages, e.g. cephalohaematoma, definite red cell aggregation was seen in the conjunctival vessels of the infant.

The case was the same if the infant was infected or suffered from erythroblastosis fetalis (Rh).

It is understandable that red cell aggregation occurs after forceps delivery or haemorrhage of the infant during delivery as this condition is comparable with trauma or haemorrhage which always cause red cell aggregation (3). But it is less evident why infants delivered by caesarean section show intravascular red cell aggregation; the delivery in such circumstances would seem even easier for the infant than in normal conditions. All mothers reveal intravascular red cell aggregation during pregnancy, and it increases considerably during childbirth (11). This per se cannot account for the red cell aggregation of an infant delivered by caesarean section as the condition is not found in infants delivered normally. The factors distinguishing an ordinary delivery from caesarean section are the greater trauma experienced by the mother, the operation, and the anaesthesia given to the mother during operation. The first question that suggests itself is whether ether, the commonest anaesthetic employed in this country for caesarean sections, produces intravascular red cell aggregation and whether, therefore, for instance the amount of ether given to the mother affects the infant and produces red cell aggregation in the infant also. It is known that ether is transferred from the mother's blood to the infant's in connection with gas metabolism in the placenta.

#### THE PRESENT INVESTIGATION

The investigation covered 8 newborn babies delivered by caesarean section at the College of Midwifery and the Second Women's Clinic — the infants were examined before  $\text{AgNO}_3$  solution was dropped into the infants' eyes as this solution per se produces red cell aggregation (1) — and 21 infants at the Children's Clinic who were anaesthetised with aether. Their ages ranged from 15 days to 11 years; the majority, however, viz. 12 children, were under 1 year of age. On examination the infants' eyes were opened with a small-sized lid opener. With a number of children to whom aether anaesthesia was given a plastic shield fixed to the skin with adhesive tape covered the eyes during anaesthesia to prevent the local effect of ether, as aether vapour coming into contact with the conjunctiva of the eye produces local red cell aggregation (2).

Conjunctival blood vessels were observed by means of a stereo-microscope  $36\times$ — $60\times$ . To the microscope was attached a tubular lamp from which the light fell obliquely on the conjunctival bulb. For photography the stereo-microscope tube was replaced with a unioocular single-objective microscope tube with an Exacta II camera mounted at the upper end. The film employed in photographing was Agfa Isopan F, speed 17/10 Din. and time of exposure 1/5000 seconds. The source of light was an Ultra-Blitz lamp whose rays were passed through a Leitz Ultropak. Photographs were taken of the conjunctiva of 7 children.

#### RESULTS

Six newborn babies born by caesarean section to mothers under ether anaesthesia revealed slight but definite red cell aggregation. The time of observation varied from 11 minutes up to 1 hour 12 minutes after birth. With one infant a definite decrease in red cell aggregation was observable in the course of the first half-hour. At the same time the colour of the infant's skin turned from cyanotic red and the infant began to react with greater liveliness to extrinsic stimuli. In these 6 cases the mother had received ether for 9—12 minutes prior to the birth of the child. An interesting observation was that two caesarean section infants born to mothers given cyclopropane as the initial anaesthesia and ether only after the child was born revealed no red cell aggregation in the conjunctival blood vessels; the red cells flowed singly without adhering to one another.

With 19 infants who were given ether and no further anaesthetic, slight red cell aggregation set in during the anaesthesia within 5—6 minutes. In the examination prior to anaesthesia none of them revealed any red cell aggregation. The investigation could not be continued on the infants under deeper anaesthesia who were operated on as operation and haemorrhage in themselves produce red cell aggregation (3). But with infants given a plaster jacket or those who had a splint renewed it was possible to examine the conjunctival vessels and intravascular red cell aggregation both under deep anaesthesia and when the patient was coming out of anaesthesia again. It was found that intravascular red cell aggregation increased with the amount of ether given to the child and decreased again fairly

TABLE

*Severity of intravascular red cell aggregation in children under aether anaesthesia*

min.	a + 0	a +	a ++
30			
25		•	•
20	•	• • • •	•
15	•	• •	•
10	• • •	•	
5	• • • •	•	
0			

rapidly when the child began to get oxygen and recover consciousness. (Table, Figs. 1—4). The severity of the aggregation is indicated in the table in the same way as in earlier publications by the author: +0 = very slight, + = moderate and ++ = fairly severe red cell aggregation. The severity of the aggregation has been compared with the time (minutes) of ether administration. However, a more effective factor seemed to be the amount of ether given and hence also the depth of the anaesthesia, but as exact values on this point are difficult to obtain the time unit was considered preferable. The local effect of ether on the conjunctival blood vessels and the red cells

Influence of ether narcosis on the formation of intravascular red cell aggregation

Fig. 1. — Age 3 ½ months. Dgn: Cheilochisis l.dx. Before ether anaesthesia, a —.

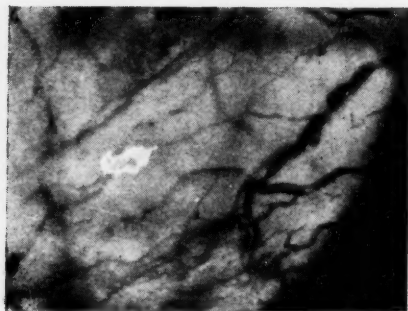
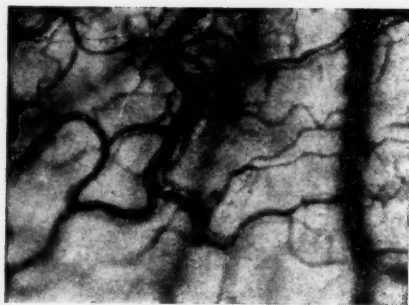


Fig. 2. — The same child. 8 min. after the commencement of ether anaesthesia, a +.

Fig. 3. — Age 1 ½ years. Dgn: Luxatio coxae cong. l.a. 15 min. after the commencement of ether anaesthesia, a +0.

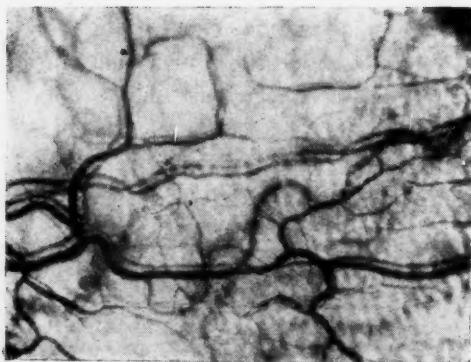
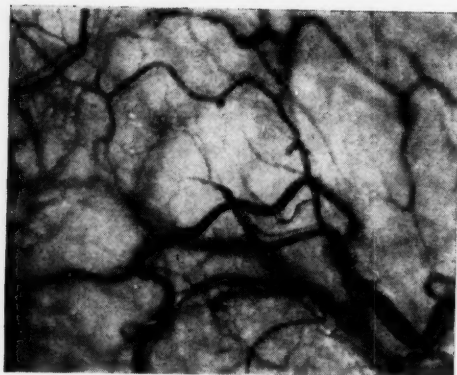


Fig. 4. — The same child. 23 min. after the commencement of ether anaesthesia, a +.

visible in them was relatively small during the anaesthesia; whether the eyes of the infant were covered with a plastic shield in addition to the customary gauze packing made hardly any difference.

Two of the children examined had definite red cell aggregation prior to anaesthesia induced by an infection; with them also a definite increase in aggregation was observed during ether anaesthesia.

#### DISCUSSION

Hueter, a German surgeon (4), found as early as 1874, on examining the blood vessels of rabbit under ether, chloroform, and alcohol anaesthesia that the red cells underwent a change and assumed irregular forms.

Knisely etc. have generally employed barbiturates (pentobarbital sodium) to anaesthetise animals. Lutz, Fulton, and Akers (8) used barbiturates (Nembutal, Narcotan) with hamsters and Thorsén and Hint (9) with rats. Knisely, Bloch, Brooks, and Warner gave horses chloral hydrate (5) and frogs urethane (7), injected subcutaneously into the lymphatic vessels. None of these substances has been found to produce red cell aggregation.

As the investigation shows definite though slight red cell aggregation to be observable shortly after ether anaesthesia is introduced it is possible that the red cell aggregation seen in infants born by caesarean section is largely produced by the ether administered to the mother. This is supported by the finding that two infants whose mothers were given cyclopropane at the beginning of the operation revealed no intravascular red cell aggregation on birth.

According to an investigation by Vara and Tiitinen (10) the  $O_2$  content of arterial blood during ether anaesthesia remains approximately the same as prior to anaesthetisation whereas the  $O_2$  content of venous blood increases. They attribute this in the first place to the inability of tissue cells during anaesthesia to receive the same amount of oxygen as normally, but they also point out that ether may affect the oxygen-binding ability of the red cells of the blood so that the oxygen is not released from the red cells in equal amounts as when the subject is not under anaesthesia. Theoretically it seems probable that the aggregation of the red cells circulating in the blood reduces the supply of oxygen as the aggregate surface area of the

oxygen-binding red cells shrinks and the flow of the bloodstream slows down. In these circumstances there is a risk that a foetus which for some other reason suffers intrauterinely from oxygen deficiency, due to ether and the resulting red cell aggregation is deprived of the minimum amount of oxygen required to sustain life.

#### SUMMARY

The blood stream and intravascular red cells can be studied directly in the conjunctival blood vessels of infants, even newborn infants. According to earlier investigations and the present continuation of that research the newborn reveal intravascular red cell aggregation if the delivery has in some way differed from the normal, for instance forceps delivery or caesarean section.

Eight newborn infants delivered by caesarean section were examined, the observation period ranging from 11 minutes to an hour and 12 minutes after the birth. The mothers of six of the infants were given aether anaesthesia, receiving aether for 9–12 minutes before the child was born. All of these children revealed definite though slight red cell aggregation. The red cell aggregation of one infant decreased markedly during the first half-hour. With two infants whose mothers were given cyclopropane at the beginning of the operation and aether only after the child was born no red cell aggregation was observable.

On the other hand, 21 infants aged 15 days to 11 years were examined before and during aether anaesthesia and while they were regaining consciousness. It was found that infants who before anaesthesia had no red cell aggregation revealed slight aggregation as soon as 5–6 minutes after the commencement of aether anaesthesia. The aggregation increased with the amount of aether given to the child and decreased again fairly rapidly when the child received oxygen. Two children suffering from an infection revealed red cell aggregation before the commencement of aether anaesthesia: their red cell aggregates increased markedly during it.

The findings are discussed.

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## LOCAL EFFECT OF AETHER AND OTHER ANAESTHETICS ON THE CONJUNCTIVAL BLOOD VESSELS AND THEIR CIRCULATING RED CELLS

by

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(Received for publication January 12, 1954)

The effect of aether anaesthesia on the various organs and tissues of the organism has been studied a great deal. It has been found that the reaction aether produces on the circulatory system is a strong dilatation of the blood vessels. On the other hand, Hueter (3) found as early as 1874 that the red cells of the rabbit assumed an irregular shape during aether, chloroform, and alcohol anaesthesia. Subsequently the effect of aether on red cells was not studied for years, and even Hueter's findings were forgotten. The possible effect of ether on red cells attracted attention again when babies born by caesarean section examined immediately after the operation revealed red cell aggregation. In fact it has been found that surgical aether anaesthesia induces general erythrocyte aggregation in the circulatory system (1).

During anaesthesia, aether is absorbed into the blood through the mucosa of the respiratory tract. The method developed by Knisely *et al.* (5), makes it easy to investigate under the microscope the blood vessels and circulating red cells in the conjunctival mucosa; blood vessels of different sizes and the flow of the red cells are distinctly seen against the white background. The present investigation uses this method to observe the blood vessels and red cells in the conjunctival mucosa under the influence of ether vapour.

Normally the conjunctival blood vessels form a fairly regular network on the mucosa. Circulating red cells wander individually

and are seen in the capillaries in single or double unbroken files. In the larger vessels the blood runs in a homogeneous mass at a speed which makes it impossible to distinguish the individual cells.

According to investigations by Knisely *et al.* (4) erythrocyte aggregation occurs after mechanical trauma and in many conditions differing from the normal such as infections, pregnancy, delivery, malignant tumours etc. An irregular flow of blood is visible in the small blood vessels and marked red cell clumps in the larger. Zilliacus (8) has observed red cell aggregates in the blood vessels of the inflamed area of the affected eye during a monocular infection when they were absent in the healthy eye.

#### THE PRESENT INVESTIGATION

The investigation was made with a stereo-microscope 36x—60x. A tubular lamp from which the light fell obliquely on the conjunctival bulb was affixed to the microscope. For photography the tube of the stereo-microscope was replaced with a uniocular single-objective microscope tube with an Exacta II camera mounted at the upper end. The source of light was an Ultra-Blitz lamp with its rays passed through a Leitz »Ultropak». The film used was Agfa Isopan, speed 17/10 Din., time of exposure 1/5000 seconds. The magnification in the film was 65x. The dilatation effect was recorded by photographing the same spot of the conjunctiva prior to and during the application of aether.

Aether and other gases were directed onto the conjunctiva of the eye by means of a current from an oxygen cylinder or by holding a gauze pad dipped in the substance close to the eye without actually touching the eye. Physiological saline, histamine and adrenaline solutions were dropped onto the conjunctiva of the eye.

The conjunctiva of the eye of 10 adult, healthy subjects was investigated both before the application of aether vapour and during the period of its effect on the blood vessels and red cells. In addition, 3 subjects with a slight infection and consequent slight red cell aggregation were also examined.

For the sake of comparison experiments were also made 1—2 times with other anaesthetics such as trichlorethylene ( $C_2HCl_3$ , Trilene ICI), ethyl chloride ( $C_2H_5Cl$ , Chloraethyl, Eichhorn), alcohol ( $C_2H_5OH$ ), divinyl aether ( $C_4H_6O$ , Vinydan, Lundbeck & Co), nitrous

## Local aeffect of ether on the conjunctival blood vessels and their circulating red cells

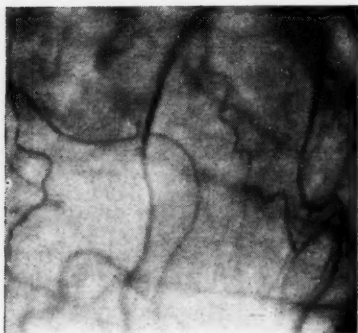


Fig. 1. — Before the application of aether vapour.

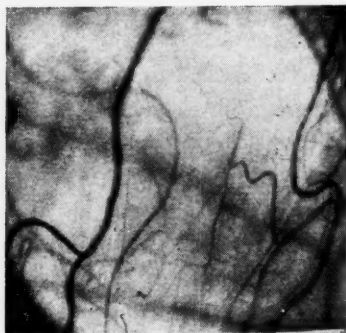


Fig. 2. — The same area. During local effect of aether.

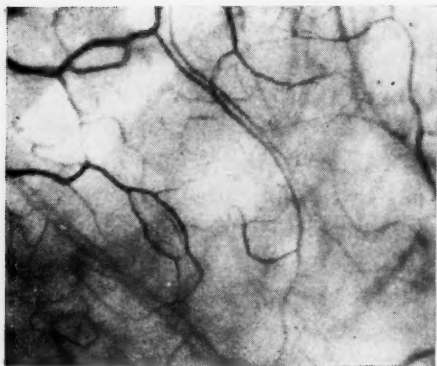


Fig. 3. — During local effect of aether.

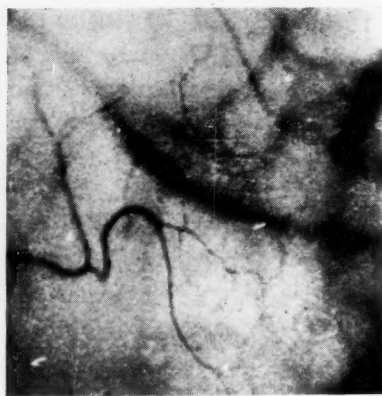


Fig. 4. — During local effect of aether.

oxide ( $N_2O$ , AGA) and pure oxygen ( $O_2$ ). The dilatation-contraction effect was also induced with histamine, dilution 1:1,000 and 1:10,000, and with adrenaline, dilution 1:10,000. Physiological saline solution was also used as control material.

As cold and heat stimuli also may induce changes in blood vessel calibre and red cell aggregation the temperatures of these different substances at the time of entering the conjunctiva of the eye were measured. Room temperature was approximately

+20°C. The temperature of the conjunctiva was measured with a Danish Jørgen A. Smith thermo-electric surface thermometer; its range was 32.7—33.5°C (10 recordings from 4 eyes). In measuring the temperature of the different vapours by an ordinary mercurial thermometer or a thermo-electric surface thermometer the indicator was held at a distance from the volatile substance, i.e. current of vapour or gauze pad, approximately equal to the distance between the substance and the eye.

Temperatures of the various vapours:

$(C_2H_5)_2O$ from gauze pad	19.0°C
$C_2HCl_3$ » » »	19.0°C
$C_2H_5Cl$ » » »	19.5°C
$C_2H_5OH$ » » »	20.0°C
$C_4H_6O$ » » »	18.0°C
$O_2$ flow 0.5 ml/min.	25.0°C
$O_2 + (C_2H_5)_2O$ » » »	25.0°C
$O_2 + C_2H_5OH$ » » »	25.5°C
$N_2O$ » » »	25.0°C

The gas flow rate in the experiments was considerably less than the rate in measuring the temperatures, and no »blow» effect was felt in the eye.

#### RESULTS

Ten healthy adults with no observable red cell aggregation prior to the test revealed marked dilatation of the blood vessels, visible macroscopically even as redness of the eye, and erythrocyte aggregation under local influence of aether vapour. Aggregates were formed immediately aether vapour came into contact with the conjunctiva of the eye, even before dilatation commenced. Simultaneously smarting was felt subjectively in the eye. After the ether disappeared the red cell aggregates dissolved relatively rapidly, in some 1—2 minutes. There was no visible change on the conjunctiva of the other eye.

With 3 adults with red cell aggregation resulting from an infection aether produced a similar dilatation and a definitely increased red cell aggregation.

The anaesthetics employed as controls, trichlorethylene, ethyl chloride, alcohol, and divinyl aether, all produced both dilatation

and a definite, fairly marked red cell aggregation. The red cell aggregation produced by nitrous oxide was considerably more slight, as was the dilatation. Pure oxygen gas produced no reaction at all.

Physiological saline at room temperature, with a slight cold stimulus applied to the conjunctiva, produced slight, barely perceptible red cell aggregation which disappeared rapidly. Heated to  $+30^{\circ}\text{C}$  it produced neither aggregation nor calibre changes in the vessels. In a test subject with slight red cell aggregation prior to the test,  $30^{\circ}\text{C}$  NaCL solution neither increased nor decreased the existing condition. Histamine in dilutions of 1:1,000 and 1:10,000, with 2—3 drops instilled on the conjunctival mucosa, produced a marked dilatation, and adrenaline, in a dilution of 1:10,000, a contraction. In solutions at room temperature both of them produced a highly transient, slight red cell aggregation comparable to the reaction produced by physiological saline at an identical temperature. If aether effect was added to their effect red cell aggregation increased momentarily very markedly.

#### DISCUSSION

Aether dropped on normal skin produces a remarkable cold stimulus by rapid evaporation. If evaporation is inhibited strong vasodilatation occurs on the skin and the test subject may feel a painful burning sensation. According to the present investigation ether evaporated from a gauze pad or directed by a current of oxygen produced on the conjunctiva also a strong dilatation of blood vessels and, subjectively, smarting but no sensation of cold.

In addition to this dilatation and even prior to it, definite, relatively strong red cell aggregation was observable in the blood vessels under the influence of the aether. It developed rapidly but also disappeared fairly rapidly, as soon as the ether vapour had had time to disappear. A current of pure oxygen did not produce this reaction. During surgical ether anaesthesia typical aggregate formation has always been observed in the circulation system in general; it increases with the amount of ether given to the patient and the depth of anaesthesia. Anaesthesia in itself, however, is not connected with the aggregates, for e.g. barbiturates (7) or »Rectanol» anaesthesia do not produce red cell aggregation.

Blood vessel calibre changes are evidently not connected with erythrocyte aggregation, as is evident from the investigations effected with histamine and adrenaline solutions. In spite of marked dilatation or contraction aggregation was very slight and transient, comparable to the reaction produced by physiological saline of identical temperature. Whenever ether effect was added marked red cell aggregation was obtained.

A theory often advanced in the literature is that the substance liberated from the tissues, e.g. due to a mechanical, thermic or chemical trauma, is the cause of aggregation. Thorsén and Hint (7) showed that thrombin at least can induce aggregation. According to Arrhenius *et al.* (2) aether vapour, on entering the alveoli of the lungs, is distributed in the ratio of 3.3: 1 between cells and plasma. In principle a similar gas diffusion to that in the alveoli of the lungs occurs on the conjunctiva of the eye when it comes into contact with aether vapour. When the aether vapour was brought into the neighbourhood of the eye in the present experiments aggregation was momentary but disappeared only gradually in the course of 1—2 minutes. Also, if the test subject for some reason had red cell aggregation before receiving aether the aggregation immediately increased considerably. No stasis formation was observable. The factor inducing aggregation in these experiments might be the aether vapour itself; the aggregates only disappear after the erythrocytes and plasma have carried away all the aether vapour that has had time to become diffused into the tissues.

Aggregation induced by aether seems to indicate that the aggregate mechanism, in this reversible initial stage at least, is a physical phenomenon. It is possible that shifts occur in the colloidal state or that changes take place in the surface tension of erythrocytes. According to Searles (6) the erythrocytes grow in size during aether anaesthesia.

Many investigators have previously suggested that aggregate formation represents a physiological protective reaction. A study of the aggregate formation on a conjunctiva induced by aether leads very readily to the thought that this may be a first stage in a patho-physiological occurrence which in extreme cases leads to postoperative thromboses and the serious complications connected with them.

## SUMMARY

The blood vessels and cells of the conjunctiva of 10 healthy adults were observed for the local reaction that occurred on the contact of aether vapour with the conjunctival mucosa. Definite dilatation was visible. But even before dilatation a marked, almost momentary aggregation of red cells was seen which disappeared in 1—2 minutes when the aether disappeared from the mucosa. In addition, with 3 adults revealing slight red cell aggregation prior to the experiment aether vapour induced a marked increase in the aggregation.

The anaesthetics employed for comparison, trichlor ethylene, ethyl chloride, alcohol, and divinyl aether, all produced a similar dilatation of the blood vessels and relatively strong aggregation of red cells. The aggregation produced by nitrous oxide was considerably less and the dilatation was fairly slight.

Pure oxygen gas produced no reaction at all.

Physiological saline solution dropped into the eye, at room temperature, produced a slight, highly transient red cell aggregation. Heated to  $+30^{\circ}\text{C}$  it produced neither aggregate formation nor calibre changes in the vessels.

Histamine 1:1,000 and 1:10,000 produced marked dilatation and adrenaline 1:10,000 a contraction. Both of them, in solutions at room temperature, produced a highly transient, slight red cell aggregation, comparable to the reaction produced by physiological saline at an identical temperature. Aether produced a marked momentary increase in red cell aggregation.

The findings are discussed.

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## HISTOCHEMICAL DEMONSTRATION OF SUCCINIC DEHYDROGENASE ACTIVITY IN HUMAN BRAIN

by

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(Received for publication January 13, 1954)

Succinic dehydrogenase is an essential enzyme in the citric acid cycle of Krebs. It converts succinic acid to fumaric acid.

The distribution of this enzyme has earlier been investigated in the brains of animals (1—4). The present writer has had an opportunity of extending these studies to human brain.

### THE PRESENT INVESTIGATION

3 specimens from normal cerebral cortex and one from cerebellum removed in connection to intracranial operations were examined for succinic dehydrogenase activity according to the histochemical method introduced by Seligman and Rutenburg (5) employing, however, neotetrazolium instead of blue tetrazolium.

### RESULTS

Both in the cerebrum and in the cerebellum of man the grey matter exhibited a fairly intense activity of succinic dehydrogenase by its reddish-purple staining whereas the white matter, the glial cells, and the capillary endothelium were unstained. In the cerebral cortex the cytoplasm of the typical pyramidal cells and of the giant pyramidal cells of Betz in the ganglionic layer (Fig. 1) was deposited by fine bluish-purple granules indicating the most intense

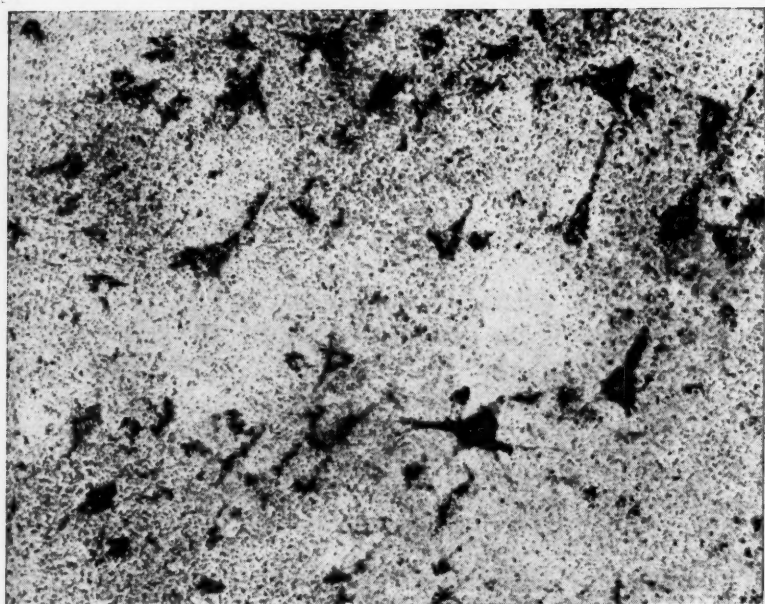


Fig. 1. — Intense succinic dehydrogenase activity in the Betz's giant pyramidal cells of human cerebrum.  $\times 220$ .

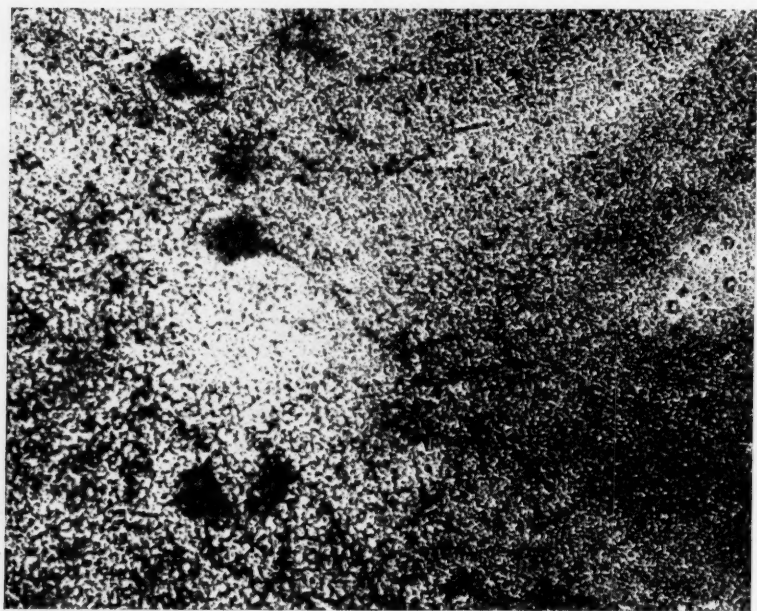


Fig. 2. — In the cerebellum of man the Purkinje's cells exhibit a more intense succinic dehydrogenase activity than the granular (at the left) and molecular (at the right) layers.  $\times 220$ .

activity. In the cerebellum the highest activity of succinic dehydrogenase was present in the perikaryon and in the dendrites of Purkinje's cells. The granular and molecular layers were somewhat less active (Fig. 2). These observations are in agreement with the results of Padykula (3) regarding the distribution of succinic dehydrogenase activity in rat brain.

*Acknowledgment.* — I am greatly indebted to Prof. Aarno Snellman, MD., who kindly placed the material at my disposal.

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## PLASMA OXIDATION-REDUCTION POTENTIAL AND THE HYPOXIC STIMULUS OF BLOOD FORMATION

by

EEVA JALAVISTO

(Received for publication March 11, 1954)

In a previous paper (5), it was shown that the plasma oxidation-reduction potential tends to rise during reticulocytosis induced in the rabbit by repeated bleedings. This finding may be interpreted in two ways. The alteration of the apparent plasma potential signals the existence of either a humoral stimulus of erythropoiesis or a chemical adaptation to increased blood formation. In the anaemic rabbit, the stimulus and response, *i.e.*, anaemic anoxia and increased rate of blood formation, exist simultaneously. Therefore, the question whether the induced anaemic hypoxia causes some chemical alteration which acts as a stimulus upon the bone marrow, or whether the increased output of reticulocytes is as such responsible for the chemical alteration manifested in a change of the plasma potential, cannot be settled. The aim of the present paper is to attack this problem by studying the effect of exposure to low atmospheric pressure upon the apparent plasma oxidation-reduction potential. If the plasma potential is seen to increase some few hours after exposure before any reticulocytosis has had time to develop, the change cannot be due to increased blood formation and may be an indication of the existence of a humoral stimulus.

### METHODS

The main part of the experiments was performed in May—June, 1952, and some supplementary experiments were carried out during the fall of 1952. As experimental animals, guinea pigs were used throughout. The

animals were kept on a diet of barley, hay and oats. Because of the season, the ascorbic acid content of the diet was probably rather poor during the main experimental period and satisfactory only in the supplementary experiments. The animals had fasted during approximately 24 hours before the blood samples were taken by heart puncture. Since it was not always possible to get arterial samples, the blood oxygen content of the sample was analysed according to a modification of the manometric method of van Slyke (6) whenever a sufficient amount of blood was obtained. The red cell count, haemoglobin concentration and percentage of reticulocytes were likewise determined from the sample.

The blood was taken in a Luer syringe sealed with mineral oil. The blood was made incoagulable by addition of a drop of 2 per cent heparin solution. The sample was then placed in two tubes under a layer of mineral oil. One of the samples was centrifuged immediately and served for measurement of the oxidation-reduction potential; the other was used for analysis of blood oxygen content.

The determination of plasma oxidation-reduction potential was made in a 1 cc Luer syringe, the piston of which was fitted with a platinum plate electrode. The tip of the syringe was dipped into a vessel filled with concentrated KCl solution. As reference electrode, a calomel electrode was used. The measurements were made with a Radiometer pH-meter, type PHM 22b. Whenever possible, two determinations with different platinum plate electrodes were made on each sample. Before each determination, the electrodes were carefully cleaned and rinsed with Sørensen's phosphate buffer solution of pH 7.4. A check of successful cleaning preceded each measurement and consisted of reading the potential when the syringe was filled with the phosphate buffer solution. If the potential did not rise to at least 240 mV, the cleansing procedure was repeated. Since the phosphate buffer represents an entirely unpoised system, it is very sensitive to impurities and is, therefore, well suited for checking the electrodes. The phosphate buffer was thereafter discarded, any remaining drop of it wiped away and the syringe filled with plasma. Air bubbles remaining in the syringe were eliminated. During this procedure, the plasma sample necessarily came in contact with atmospheric air. Since the oxygen tension in arterial blood comes close to that in the air, this was not considered to be a fault. The potential stabilized rapidly as a rule; after 10 minutes the change was so slow that the final reading was made. As pointed out by Bembé and Dietrich (2), the anaerobic potential requires a much longer time for equilibration and is, therefore, unsuited for serial determinations.

The methodical error of duplicate potential measurements with two electrodes was determined from the equation  $S = \sqrt{\frac{\sum \Delta^2}{2n}}$  where  $\Delta$  = difference of the values with electrodes 1 and 2 and  $n$  = number of observations.  $S$  was found to be 5.6 mV in 53 duplicate determinations. This compares favorably with the differences observed between different electrode potentials in earlier investigations (2).

The plasma oxidation-reduction potential was measured in three experimental groups of guinea pigs:

1. In 22 guinea pigs, serving as controls.
2. In 13 animals, two to four hours after exposure to low pressure (360—420 mm Hg) for three hours.
3. In 13 animals, three to four days after the first heart puncture.

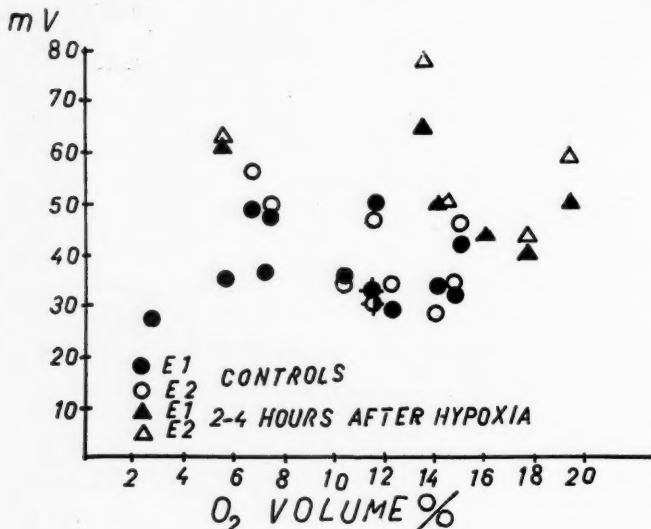


Fig. 1.

#### RESULTS

*Plasma Potential and Oxygen Content of Blood.* — In earlier experiments (5) the impression was gained that it was necessary to make all measurements of plasma potential on arterial plasma in order to obtain consistent results. According to Bembé and Dietrich (2), too, the variations in the potential are greater in venous than in arterial samples. Therefore, since it was not always possible to get arterial blood by heart puncture in guinea pigs, the eventual influence of blood oxygen content upon the apparent oxidation-reduction potential was studied in 18 samples. No interdependence of these two variables was seen, as shown in Fig. 1, in which the plasma potential is plotted against oxygen content of the blood sample. This lack of correlation may be a result of the fact that the plasma samples when introduced into the electrode syringe become practically equilibrated with atmospheric air.

*Exposure to Low Barometric Pressure and Plasma Potential.* — It may be noted in Fig. 1 that most of the plasma potential values recorded after exposure to low pressure lie above the control values; but the determinations with analysis of the oxygen content are too few for definite conclusions. In Fig. 2 all determinations are seen. The values for both electrodes are given separately, and the determinations made in December are marked with circles. As seen from

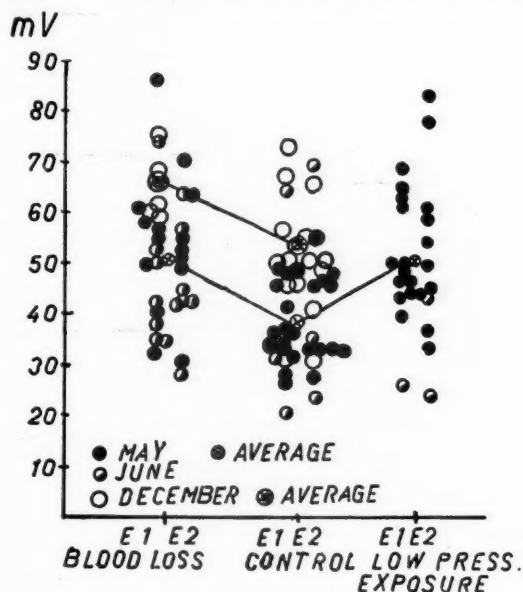


Fig. 2.

the figure, the scatter of the values is rather great in both normal controls and the other experimental groups. This scatter is somewhat reduced if determinations made only in May are taken into account. On the whole, the values for control samples seem to be lower than those for the plasma samples taken two to three hours after exposure to low pressure, the mean potential value recorded with electrode 1 being  $38 \pm 2.1$  for controls and  $51 \pm 2.9$  for those subjected to low pressure. The difference,  $13 \pm 3.8$ , is statistically significant at the 0.27 per cent confidence level.

*Plasma Potential after Bleeding.* — In those guinea pigs subjected to heart puncture three to four days prior to the plasma potential determinations and in which a definite reticulocytosis was

TABLE 1

	May			December	
	Controls	After Low Pressure	After Bleeding	Controls	After Bleeding
Er, mill/cu mm	4.90 ± 0.11	4.53 ± 0.19	— —	— —	— —
Hgt, g/100 cc	11.8 ± 0.20	11.1 ± 0.39	9.2 ± 0.9	— —	— —
R, %	0.93 ± 0.13	0.99 ± 0.21	3.7 ± 0.9	— —	— —
mV El. 1	38 ± 2.4 (n=13)	51 ± 2.9 (n=10)	55 ± 4.8	0.8	— —
mV El. 1 and 2	39 ± 2.0 (n=23)	52 ± 3.1 (n=19)	54 ± 3.2 (n=15)	54 ± 5.1 (n=8)	65 ± 3.3 (n=5)

TABLE 2

STATISTICAL EVALUATION OF THE DIFFERENCES

Difference	Electrode 1			Electrodes 1 and 2		
	mV	t	P	mV	t	P
Controls, May—Dec.	16 ± 5.6	2.9	1% > P > 0.27%	—	—	—
Controls, May — after hypoxia, May	13 ± 3.8	3.4	0.27%	13 ± 3.7	3.6	< 0.27%
Controls, May — after bleeding, May	17 ± 5.3	3.1	1% > P > 0.27%	15 ± 3.8	3.9	< 0.27%
Controls, Dec. — after bleeding, Dec.	11 ± 6.1	1.8	> 5%	—	—	—

noted, the plasma potential was elevated by an amount approximating that seen in those samples taken two to three hours after exposure to low pressure. Between the degree of reticulocytosis and the plasma potential, no obvious proportionality could be observed, but the highest reticulocyte percentage coincided with the highest plasma potential. The mean values are given in Table 1, and the statistical evaluation of the differences is shown in Table 2. In December, the potential level is higher in the controls but again slightly more so in those samples taken three to four days after the first heart puncture. The number of experiments is too small for comparison. Unfortunately, no experiments with exposure to low pressure were made during that season.

#### DISCUSSION

The difficulties encountered in the measurement and interpretation of oxidation-reduction potentials are well known. Even in reversible organic oxidation-reduction systems, the equilibration time of electrode potentials may vary, and small impurities of electrodes may totally invalidate the results, especially when working with dilute, weakly poised systems.

In biological fluids that do not form true reversible oxidation-reduction systems, the difficulties are even more pronounced. The accuracy of the measurements presented in this study has been reached only with extreme care in regard to the cleanliness of the electrodes and uniformity in handling the samples, yet it is not quite as great as would be desirable for the delicate problem dealt with here. The results can, therefore, be considered only as guides for framing a working hypothesis concerning the nature of the hypoxic stimulus of erythropoiesis. In a previous paper (4), it was pointed out that the existence of a humoral stimulus may not be bound to any specific chemical system or reaction but may appear in connexion with a variety of oxidation-reduction processes taking place in the blood and being manifested in a change of the apparent oxidation-reduction potential of the blood plasma.

The results of the present study do not, of course, prove the validity of such a hypothesis, but they do not contradict it. The question whether the change in plasma potential observed in post-haemorrhagic anaemia (5) is due to the increased blood formation

or to the hypoxic humoral stimulus remains partly unsolved. The present results show, however, that the change in the plasma potential is not necessarily bound to the increased blood formation and may consequently represent the hypoxic stimulus. On the other hand, the higher potential in the post-exposure period cannot be proved to depend on the existence of the humoral hypoxic erythropoietic stimulus. The exposure to low pressure represents a stress, that may induce humoral reactions with possible effects on the oxidation-reduction processes, but perhaps without stimulating the erythron. Since two to three hours elapsed after exposure, an adrenaline effect is not likely to be responsible for the difference.

It is interesting to note the difference in the potential level between determinations made in May and those made in December. In view of the fact that many of the vitamins form oxidation-reduction systems, it is probable that the nutritional status is responsible for the differences. Seasonal differences in potential of the same order of magnitude are reported in tuberculous patients (1).

#### SUMMARY

1. Measurements of the (apparent) oxidation-reduction potential in blood plasma of guinea pigs were performed in normal animals and after the animals were exposed to low pressure.

2. Samples taken two to three hours after exposure of the guinea pigs to low pressure of 360–420 mm Hg showed on an average a slightly higher plasma potential than normal blood samples:  $38 \pm 2.1$  mV (controls) and  $51 \pm 2.9$  mV (after exposure), or given as normal hydrogen electrode potentials:  $284 \pm 2.1$  and  $297 \pm 2.9$  mV, respectively.

3. A similar average increase in the plasma potential was noted in samples taken from animals three to four days after the first heart puncture, when a moderate reticulocytosis had developed.

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## THE PARADOXICAL EFFECT OF GIANT INTRAVENOUS INSULIN DOSES ON RABBITS

by

ESKO K. NÄÄTÄNEN

(Received for publication March 18, 1954)

While engaged in research on the haemo-encephalic barrier, I once discovered that even such an enormous dose of insulin as 1000 international units did not always cause shock in rabbits. As the ineffectiveness of insulin seemed to be the only reasonable explanation to this phenomenon, insulin manufactured by another laboratory was used in further studies. The effect of extra heavy intravenous doses of insulin was still slight, but in small doses both kinds of insulin were quite effective. This paradoxical phenomenon, i.e. small doses more effective than large ones, was thus in no way connected with the quality of insulin and the explanation had to be found elsewhere. (Most of the insulin used manufactured and contributed by Medica).

I have been unable to find any work in the literature in which insulin had been used in the same quantity. Bjerner and Swenson (1) had observed the same phenomenon when using much smaller doses of insulin. They found that intravenous doses of 50 international units of insulin per kg affected the general condition and blood sugar of a rabbit less than did four or ten international units per kg when injected intravenously or subcutaneously. Fifty international units of insulin when injected intravenously did not cause any reaction in 15 of 25 rabbits; the rest had slight cramps. All the animals recovered, but two of them received injections of glucose to stop the effect of the insulin. The largest dose of insulin

reduced the sugar content of the blood slowly. The smallest dose of four international units seemed to reduce the blood sugar level most quickly.

Among different rabbits the reaction to insulin varied greatly. The experiments were performed three times on each of five rabbits, using different amounts of insulin (4, 10 and 50 international units). The rabbits received their ordinary feed the whole time.

Bjerner and Swensson (1) could not explain their observations.

As no explanation could be found in the literature as to this peculiar phenomenon, I performed several experiments. A detailed description not being called for, in my opinion, I will present only the essential facts.

#### OWN OBSERVATIONS

Tests were performed on 46 male rabbits, each of them weighing circa 2 kg. They received their ordinary feed the whole time (cabbage, rutabaga and hay). Observations were made in the afternoos; injections of insulin were given at 1 pm. Most of the rabbits received intravenous injections, the doses varying from 10 to 500 international units per kg. Large doses of insulin were injected very slowly. For the sake of comparison, some rabbits received the same doses of insulin subcutaneously or intraperitoneally. The effect of insulin on the blood sugar content was studied according to the method used by Crecelius and Seifert. The blood tests were taken just before and one, two and three hours after the insulin injections.

Bjerner and Swensson (1) performed several experiments with different doses of insulin on each rabbit. In my opinion the previous insulin injections, especially when large doses are used, may affect the results of later tests. Therefore, in my experiments, each of the doses of insulin was injected into different rabbits. As the effect of insulin on different rabbits varies, all the tests were carried out on a number of rabbits to eliminate the influence of individual differences.

For the sake of comparison, rats and guinea-pigs were injected with different doses of insulin subcutaneously or intraperitoneally.

#### THE EFFECT OF INSULIN ON RABBITS

Soon after a dose of insulin, the rabbit usually sits quietly in its corner, often one ear hanging. When the effect of insulin

grows stronger, its legs give way and it lies on its stomach, later on its side. During the cramps the animal moves its legs as if running or rotates around its vertical axis. In the interval between cramps the rabbit usually lies on its side.

The effect of insulin on different rabbits varies greatly. Various experiments showed, however, that a small dose of insulin generally produced a shock more quickly and more often than a heavy dose. Some rabbits could stand as much as 400 or 500 international units of insulin per kg without cramps, though they usually showed reluctance to move.

The blood sugar level as well as the general condition of the animal was usually more affected by the small doses of insulin than by the large ones. Fig. 1 shows the effect of three different doses of insulin on the blood sugar in rabbits 1, 2 and 3. The first rabbit received 400 units per kg and the second one 10 units per kg intravenously. In the third 400 units of insulin per kg were injected

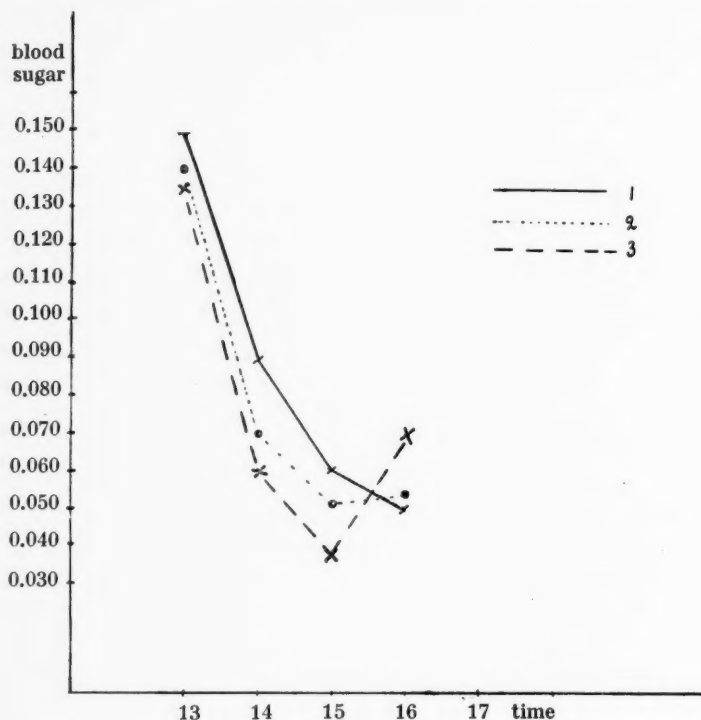


Fig. 1

subcutaneously. We find that in No. 2 the blood sugar decreased faster than in No. 1, which received an enormous dose of insulin.

No. 3 had the most noticeable decrease in blood sugar, caused by a large dose of insulin, which was injected subcutaneously and which, therefore, was absorbed slowly into the blood. Rabbits No. 1 and 2 could stand their doses of insulin rather well but No. 3 had to be given an injection of glucose after the test. The effect of insulin on the blood sugar content of No. 2 and No. 3 is rather similar, but in the case of No. 1, compared with the others, the decrease is slower. It is quite apparent that some retarding factor starts to operate when large amounts of insulin are injected into the blood. Small quantities do not seem to be able to start the action of this factor.

What then is this factor which retards the rate of decrease in the blood sugar? Houssey and Magenta (2), proved in 1924 that hypophysectomized animals are oversensitive to insulin. Would the pituitary cause this paradoxical effect of giant doses of insulin?

#### PITUITARY GLAND AND GIANT DOSES OF INSULIN

In order to find out what role the pituitary plays in this paradoxical effect of giant doses of insulin, a group of rabbits had lesions of varying degrees inflicted on the pituitary, after which the effect of large doses of insulin on these animals was studied. Fig. 2 shows the effect on the blood sugar of one of these groups of an intravenous injection of 400 units of insulin per kg. Rabbits 4, 5 and 6 received ether anaesthesia at 10 a.m. and the pituitary was damaged with a dentist's drill through the soft palate. The extent of the lesions thus caused was ascertained in postmortem examinations. Rabbit K was used as a control animal and it received the same treatment without having the pituitary damaged. The blood sugar curve of rabbit K was similar to that of rabbit No. 1, which received an intravenous injection of 400 units of insulin per kg as shown in Fig. 1. This proves that anaesthesia and the operation alone did not affect the blood sugar. We notice that the blood sugar of the three rabbits which had lesions on the pituitary decreased more quickly than that of rabbit K. According to the diagrammatic drawings, one could presume that rabbit No. 4 had suffered the slightest damage to the pituitary and No. 6 severest.

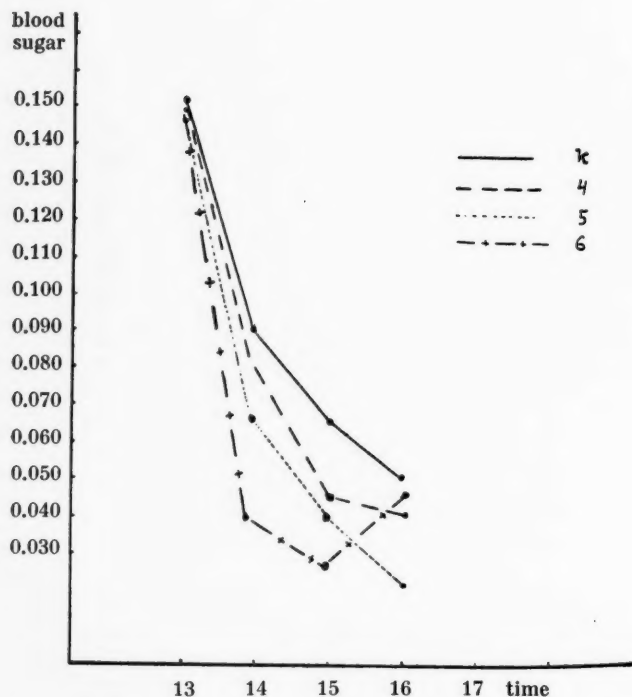


Fig. 2

The postmortem examination proved this presumption quite accurate. No. 4 had the pituitary only slightly damaged; in No. 6 it could hardly have been active any longer. There was minimal change in the general condition of No. 4 after the injection of insulin; it was tired but seemingly in quite good condition during the five hours the experiment lasted. No. 5, deemed to have a relatively badly damaged pituitary, was in good condition after the operation; but in less than two hours after the injection of insulin, it had severe cramps. As it did not show any signs of recovery, it had to be killed  $3\frac{1}{2}$  hours after the injection. No. 6 also seemed strong before the insulin injection, but 2 hours later it likewise had cramps.

It appears that, when the pituitary is damaged, the ability of rabbits to stand the effect of heavy doses of insulin decreases in proportion to the extent of the lesions. If the pituitary is damaged, there is a rapid decrease in blood sugar, the general condition

deteriorates and the rabbit suffers cramps. Apparently the rabbits used in this experiment would have died if they had not been killed.

It is evident that the ability of rabbits to stand extra heavy doses of insulin depends on the activity of the pituitary. A large amount of insulin in the blood causes a state of alarm in the organism, during which the pituitary, probably stimulating the suprarenal glands, causes an increase in the blood sugar. Usually we are able to reduce blood sugar rapidly and cause a shock readily by using small amounts of insulin. There must be a comparatively large amount of insulin in the blood to cause an alarm reaction. A giant dose of insulin injected subcutaneously or intraperitoneally does not cause this reaction as apparently insulin is absorbed into the blood in far too small amounts. It was also observed that no paradoxical reaction occurred when rats and guinea-pigs received insulin injections subcutaneously or intraperitoneally. When different doses were given to these animals, it was observed, as expected, that the larger doses had a stronger effect than the smaller ones.

#### THE EFFECT OF RENEWED GIANT DOSES

Because of the paradoxical reaction described in the foregoing, a rabbit can usually stand a heavy dose of insulin better than a small dose. If a rabbit receives more than one giant dose of insulin during the course of several days, its endurance rapidly deteriorates, especially if the intervening time is only a few days. Fig. 3 shows the effect of renewed giant doses of insulin on blood sugar. Rabbit No. 7 received 200 units of insulin per kg three times in one week before the examination of blood sugar. The diagram shows that, after such a pre-treatment, an injection of 400 units per kg causes a more rapid decrease in blood sugar. For the sake of comparison, the blood sugar curve of two more rabbits is shown in Fig. 3. In both rabbits, which two days previously had received 200 units per kg, the injection of 400 units per kg did not cause a decrease of blood sugar equally rapidly and sharply, but the blood sugar decreased more slowly, reaching a very low level. All these rabbits were so weak that they had to be destroyed.

Thus the diagram shows that in rabbits No. 8 and 9, which previously had received one insulin injection, the retarding effect

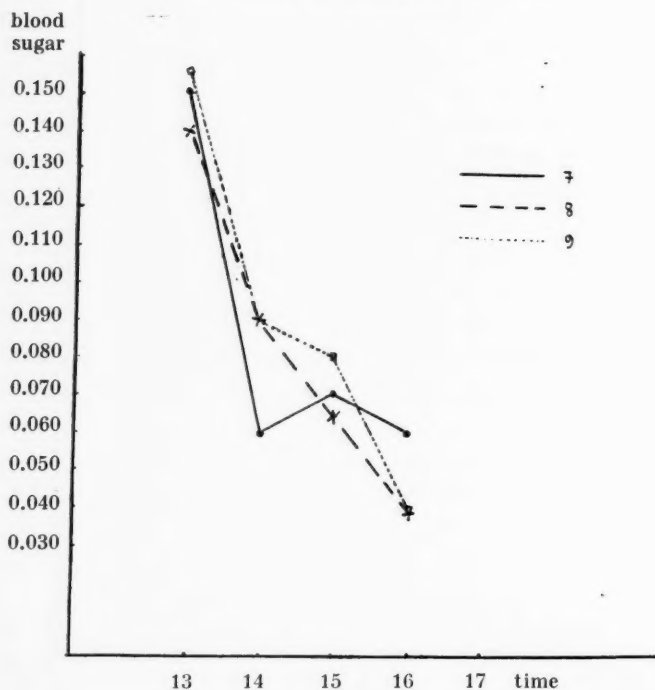


Fig. 3

produced by the pituitary still prevents a rapid decrease of blood sugar; but in rabbit No. 7, which received several injections, the blood sugar decreases more rapidly and sharply during the first hour presumably because previous injections of insulin have caused exhaustion in cells, which counteract the effect of insulin. Over-exertion, caused by the renewed insulin injections, may cause changes in these cells and thus give us, in all probability, a chance to find out which group of cells of the pituitary really causes this astounding ability of rabbits to stand giant doses of insulin. In my next work I shall deal with this question.

#### THE EFFECT OF CASTRATION

Since it has been established that castration causes changes in the cells of the pituitary, there were grounds for trying to discover what the effect of castration was on the ability of rabbits to stand giant doses of insulin. Accordingly, two large rabbits (weighing

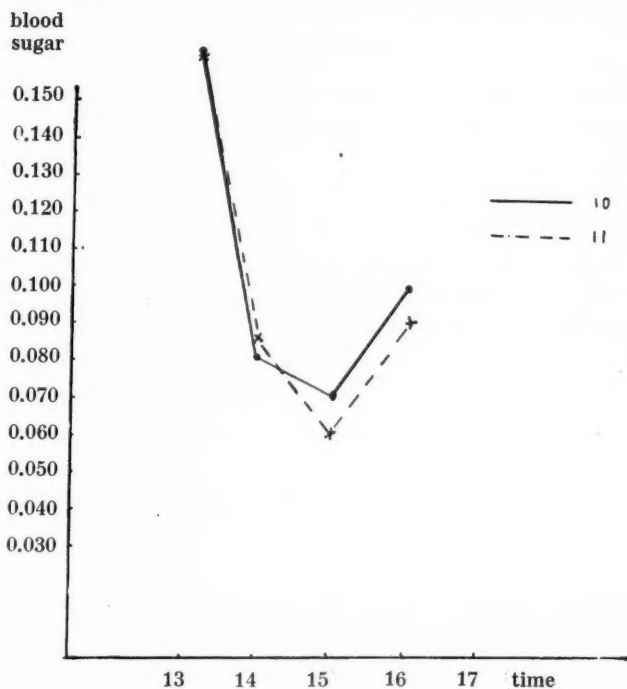


Fig. 4

3250 g and 3750 g) were castrated. One week after the operation, both rabbits received one intravenous injection of 400 units of insulin per kg each, and the blood sugar was examined as previously. Fig. 4 shows that the blood sugar of both rabbits decreases comparatively slowly. Three hours after the insulin injection a rather noticeable increase in blood sugar attracted attention. Further examinations will show whether this increase in blood sugar in both castrated rabbits is produced by some fortuitous factor, or by some changes in the endocrine glands caused by the castration. Both rabbits died, one in  $3\frac{1}{2}$  hours and the other one 10 hours after the insulin injection. No certain conclusions can be drawn, as the material collected so far is inadequate; it appears, however, that castration one week prior to the test does not essentially affect the decrease in blood sugar caused by the giant doses of insulin.

## SUMMARY

The ability of rabbits to stand insulin injections differs individually. Studies with numerous animals have led to the following conclusions:

A rabbit usually seems to be able to stand a giant intravenous dose of insulin (e.g. 400 units per kg) better than a very small dose (e.g. 10 units per kg). Cramps, if any, caused by a giant dose of insulin occur generally several hours after the injection. A small dose of insulin causes cramps earlier, often  $1\frac{1}{2}$ —2 hours after the injection.

This astonishing phenomenon may be explained by the fact that the decrease in blood sugar seems to be slower after heavy doses of insulin than after small ones.

It is obvious that this phenomenon is caused by the action of the pituitary. If an animal has had its pituitary damaged, its ability to stand giant doses of insulin lessens and the content of sugar in the blood decreases more rapidly after an injection.

The ability of rabbits to stand a heavy dose of insulin deteriorates considerably if they have received large doses of insulin previously, especially if injections are given at short intervals. There is also a more rapid decrease of blood sugar in these cases. This may be due to the exhaustion of those cells which are responsible for the reaction of the pituitary. To start this reaction, which retards the rate of decrease in the blood sugar, there obviously must be a large amount of insulin in the blood. A small amount cannot cause this reaction, and therefore, the blood sugar decreases rapidly.

Future histological research may show what group of cells of the pituitary accounts for the astonishing ability of rabbits to endure giant doses of insulin.

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## THE EFFECT OF EPHYNAL ON THE POSTHAEMORRHAGIC RETICULOCYTOSIS AND REGENERATION OF BLOOD IN RABBITS

by

EEVA JALAVISTO

(Received for publication March 27, 1954)

The nature of the fundamental stimulus of erythropoiesis is not known. The question has recently been reviewed by Grant and Root (7). The authors conclude that there might be more than one mechanism responsible for normal stimulation and regulation of erythropoiesis, a true stimulation being achieved experimentally by three conditions: anoxic anoxia, anaemic anoxia and cobalt.

It has become evident that lowered oxygen tension in the bone marrow cannot directly stimulate erythropoiesis (1, 7, 15, 19). It has therefore been postulated that anoxia induces humoral alterations e.g. formation of hemopoietines demonstrated first by Carnot and Deflandre (5). There is no knowledge of the nature of this agency, except that it may be destroyed by contact with oxygen (14).

The effect of cobalt has been related to interference with respiration of immature red cells in the bone marrow (actually demonstrated to occur *in vitro*) and prevented by administration of ascorbic acid (2). The diminution of the metabolism of the reticulocytes is then supposed to cause their discharge into the circulation and replacement by a new generation of cells. Since cobalt is a toxic, foreign substance it might be more appropriate to search for a physiological agency which likewise would interfere with oxidative processes.  $\alpha$ -tocopherol is known as a biological nontoxic anti-oxidant. According to Houchie (9)  $\alpha$ -tocopherol decreases the oxygen consumption of brain homogenates.

The aim of this paper was to determine whether a stimulation of the erythron could be demonstrated as a result of administration of a  $\alpha$ -tocopherol preparation «Ephynal» (Roche). Since the erythrocyte level is very effectively adjusted, it was thought that the degree of posthaemorrhagic reticulocytosis would perhaps be a more sensitive indicator for the existence of an erythropoietic effect than the incidence of polycythaemia.

#### METHODS AND MATERIAL

The experiments were performed on 8 rabbits during the winter season 1953—1954. The left carotid arteries of the rabbits were exteriorized in preliminary operations and the bleeding of the animals was effected by puncturing the carotid loops. This procedure was deemed necessary in order to be able to remove repeatedly exactly measured amounts of blood. One experimental period lasted 26 days during which blood samples from marginal ear vein for enumeration of the erythrocytes, determination of haemoglobin and percentage of reticulocytes were taken according to a rigid scheme. During one experimental period 4 bleedings were undertaken one on the second day and 3, 14 and 17 days thereafter. The amounts of blood taken at each puncture varied between 12—20 ccm, the removal of exactly same amounts being attempted in control experiments and under administration of ephynal. In the ephynal series the rabbits received daily (except on the second, 9<sup>th</sup> and 16<sup>th</sup> day) 15 mg and in three experiments 30 mg of tocopherol (Ephynal «Roche») as intramuscular injections. The number of control experiments was 16, that of the experiments with administration of ephynal 12.

#### RESULTS

*Haemoglobin and Erythrocytes.* — The initial concentration of haemoglobin in the control series and in the ephynal-treated cases was exactly the same viz. 10.3 g/100 ml on an average. The decrease was maximal on 4th day after the first bleeding (on the day following the second removal of blood) and the difference from initial value was again exactly the same in controls and ephynal-treated rabbits viz. 1.9 g/100 cc. The series may therefore be considered as well suited for comparison of the rates of regeneration. As seen from figure 1a, there is no difference between controls and ephynal-treated in the rate of haemoglobin regeneration during the 11 days period of observation. As often pointed out (3, 8) the anoxic stimulus does not primarily affect the synthesis of haemoglobin, the color

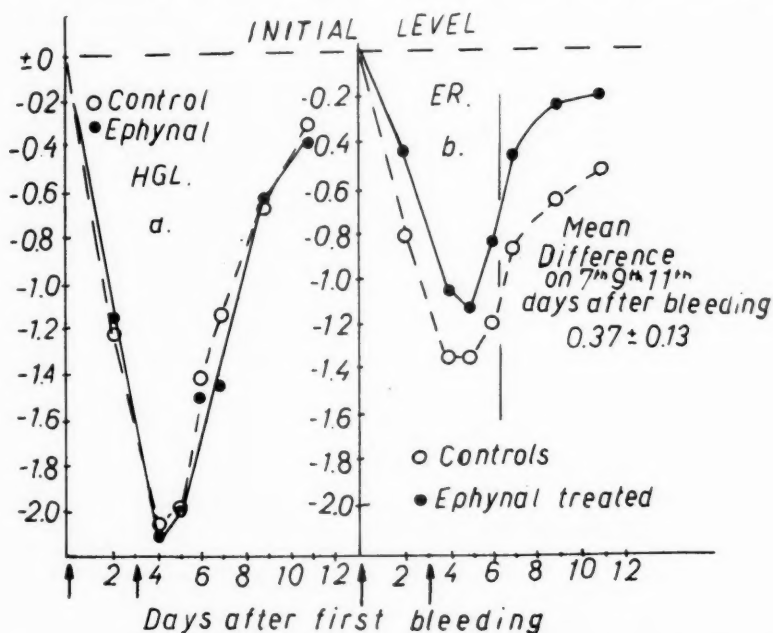


Fig. 1. — Difference from initial value of haemoglobin a) and erythrocytes b) in control and ephynal treated rabbits after removal of blood on the days marked with arrows.

index often decreasing during an acute anoxic erythrocytosis. The absence of any effect of ephynal on haemoglobin regeneration cannot, therefore, be interpreted as an indication of lack of stimulation of erythropoiesis. Indeed, if the red cell count and the percentage curves of reticulocytes are examined, a stimulating effect seems to manifest itself.

The initial value of erythrocytes is practically the same both in the control series,  $Er = 5.60$  mill/cu mm, and the ephynal treated,  $Et = 5.30$  mill/cu mm. The lowest value is encountered on the 4—5<sup>th</sup> day after first bleeding in the control series and on the 5<sup>th</sup> day in the ephynal-treated. Numerically the difference from initial value is slightly less in the group of ephynal-treated but the difference is not significant during any of the 6 first days after bleeding. The difference of the 7<sup>th</sup>, 9<sup>th</sup> and 11<sup>th</sup> day taken together amounts on an average to  $0.37 \pm 0.13$  which corresponds to a 0.5 per cent level of confidence, and may consequently be regarded as probably significant (Figure 1b).

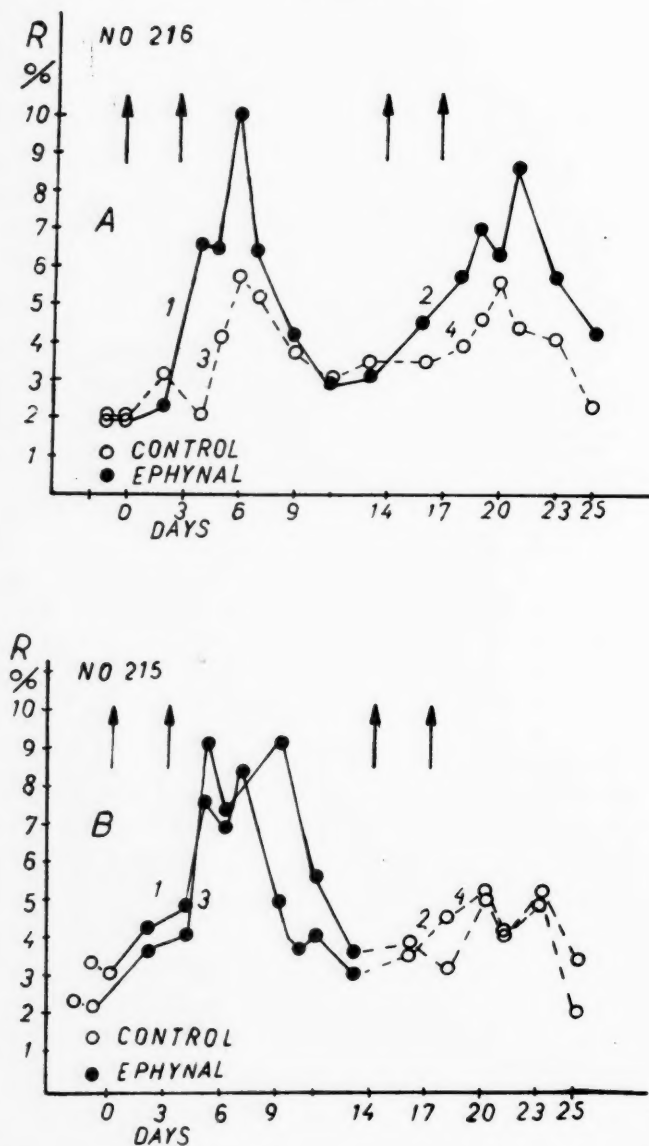


Fig. 2. — Reticulocyte responses during control and Ephynal medication periods.

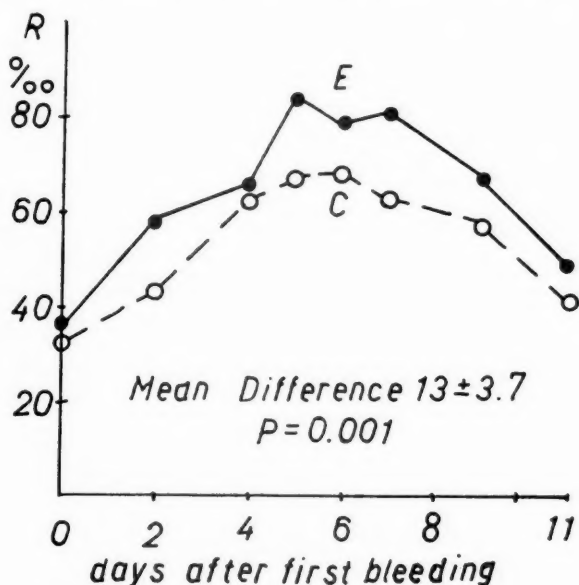


Fig. 3. — Post-haemorrhagic reticulocytosis in control and Ephynal experiments. Average values.

*Reticulocytes.* — In conformity with the results concerning the erythrocyte count slightly greater reticulocyte responses are observed in the series treated with Ephynal. In figure 2a and 2b the reticulocyte responses in four control periods and 4 periods with administration of ephynal are seen. The curves are numbered in the order of the sequence of the experiments: in 2 A two similar eleven day periods were made in succession and in 2 B the period with ephynal-medication preceded the control-period. The bleedings are marked with arrows. As seen from the figures the reticulocytosis is less marked in the controls as during the influence of ephynal. Single cases cannot, however, be taken as evidence. In three of the 8 rabbits no effect was seen. The material was therefore compared as a whole. In fig. 3 the average values of reticulocytes are given. The initial mean values are the same in controls and ephynal-treated rabbits but already on the second day an elevated value is encountered in those receiving ephynal. The difference is only probable on any single day but taken together the difference in the

output of reticulocytes during the 11 days period following the first blood tapping is highly significant  $13 \pm 3.7\%$ . The probability of the difference to occur as a random variation is 0.001.

#### DISCUSSION

The observed stimulating effect of ephynal upon erythropoiesis is not great. However, the lack of any effect upon the regeneration of haemoglobin and the exact coincidence of the haemoglobin control and ephynal values may be taken as a proof for the similarity of experimental conditions in the control and the ephynal-medication series. The parallel observation of slightly increased regeneration rate in the erythrocytes and the greater output of reticulocytes support the evidence of statistical significance. The fact that an effect is noted both in reticulocytes and erythrocytes is important. The greater percentage of reticulocytes could otherwise be interpreted as due to slower maturation rate (6, 17) because of the effect of  $\alpha$ -tocopherol as an antioxydant. Now this interpretation seems not very likely, but of course, it cannot be entirely ruled out. Direct experiments for elucidation of the effect of  $\alpha$ -tocopherol on the maturation of reticulocytes are planned. Experiments with higher doses of ephynal in order eventually to provoke a greater effect are likewise going on.

It is too early to speculate upon the mechanism of action of ephynal on the regeneration of blood. However, it is rather curious to note that an agent most likely not concerned with the oxygen carrying capacity of haemoglobin simulates the effect of anoxia probably by its function as an antioxydant and an oxidation-reduction system. The concept of the anoxic stimulus as a humoral factor affecting the oxidation reduction equilibrium in blood (9, 10, 11) seems therefore to gain support by the results exposed in this study.

It may, however be pointed out that this is not the only possibility. It has e.g. been claimed (4) that  $\alpha$ -tocopherol would cause a contraction of the spleen. Ruhenstroth-Bauer (18) on the other hand has shown that the posthaemorrhagic reticulocytosis is diminished after splenectomy and a stimulating effect of splenic blood on erythropoiesis has been noted by Istamanova and Tschilipenko (10). The effect on erythrocytes and the percentage of reticulocytes in this study could therefore represent a reaction

on outpouring of some haemopoietic principle from the spleen, but the evidence of the existence of such a substance is very scarce. However, experiments on the effect of Ephynal on the posthaemorrhagic reticulocytosis in splenectomized animals, and a study of the size of the spleen after injections of Ephynal in different animals are going on.

#### SUMMARY

1. The effect of  $\alpha$ -tocopherol (»Ephynal«-Roche) on the percentage of reticulocytes and the regeneration of haemoglobin and erythrocytes after bleeding are studied in 8 rabbits.

2. The regeneration rate of haemoglobin is not affected by Ephynal.

3. A slight speeding up of regeneration of erythrocytes together with a slight but highly significant increase in the output of reticulocytes is noted during administration of Ephynal.

4. The effect is tentatively referred to the property of  $\alpha$ -tocopherol as an antioxydant affecting the oxidation reduction systems in blood. The effect of ephynal is paralleled to the phenomenon of anoxic erythrocytosis.

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## VERGLEICHENDE VERSUCHE ÜBER DIE WIRKUNG VERSCHIEDENER ATMUNGSSTIMULANSEN

### II TEIL

von

EINO V. VENHO, OSMO VARTIAINEN und MATTI VAPAAVUORI

(Am 31. 3. 54 bei der Schriftleitung eingegangen)

Im zweiten Teil unserer vergleichenden Versuche über atmungsstimulierende Stoffe behandeln wir Pyridin- $\beta$ -Carbonsäurediäthylamid oder Coramin, Pentamethylentetrazol oder Cardiazol, Ortophtalsäurebisdiäthylamid oder Neospiran, 3,5-Dimethyl-Isoxazol-4-Carbonsäurediäthylamid oder Cycliton und Methylisopropylhexenon oder Hexeton. Die Untersuchungstechnik ist im ersten Teil der Arbeit beschrieben (14).

#### DAS CORAMIN

Das Coramin<sup>1</sup> oder Pyridin- $\beta$ -Carbonsäurediäthylamid wird als Atmungsstimulans und zentrales Analepticum angewandt. Seine pharmakologischen Wirkungen betreffen in der Hauptsache das Zentralnervensystem und erinnern ausser an die Wirkungen des Kampfers auch an diejenigen des Nikotins. Es wirkt nicht nur stimulierend sondern in grossen Dosen auch deprimierend. Die Stimulation der Atmung zeigt sich schon bei Gaben, die nur wenig andere Wirkungen besitzen. Die toxischen Dosen verursachen Krämpfe und führen durch Atmungslähmung zum Tode. Das Coramin wirkt erheblich besser auf die deprimierte als auf die normale Atmung. Die Auffassungen über den Antagonismus gegen Narcotica sind verschieden, und derselbe ist wahrscheinlich effektiver,

<sup>1</sup> Ciba Aktiengesellschaft, Basel.

wenn die zentrale Depression durch Morphin oder Inhalationsnarcotica hervorgerufen worden ist, und nicht durch grosse Barbituratdosen. In grossen Dosen kann die lähmende Wirkung des Coramins die zentrale Depression vermehren. Seine therapeutische Breite ist jedoch beträchtlich, und seine Wirkung soll länger anhalten als z.B. die des Cardiazols (12, 5).

Die Wirkung des Coramins auf die durch Morphin herabgesetzte Atmung beim Kaninchen haben wir in 12 Versuchen untersucht. Die intravenös verabreichte Morphindosis war in allen Versuchen gleich, nämlich 4 mg/kg. Die intravenösen Coramindosen variierten von 5–30 mg/kg. Bei Dosen von 10 mg war die Wirkung schwach, und eine nennenswerte Besserung der Atmung war nicht wahrzunehmen. Die Dosis von 15 mg hatte in zwei Versuchen nur schwache Wirkung, in einem hingegen sehr starke, wobei das Atmungsvolumen für einige Minuten beträchtlich über das Normale stieg. Die Dosen von 20–30 mg hatten in allen Versuchen kräftige Wirkung, und das Atmungsvolumen kehrte auf das normale Niveau zurück (Abb. 1). Bei sämtlichen Versuchen stieg die Atmungsfrequenz der

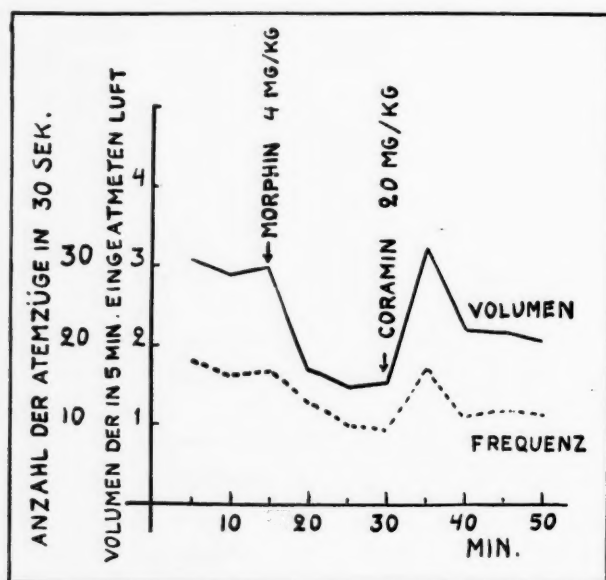


Abb. 1. — Wirkung des Coramins auf die durch Morphin herabgesetzte Atmung des Kaninchens. Gewicht des Versuchstiers 2.5 kg. Morphinhydrochlorid 4 mg/kg intravenös und danach Coramin 20 mg/kg intravenös.

Volumenzunahme entsprechend. Die Wirkung hielt je nach der Dosis 10—25 Minuten lang an. Bei Dosen von 30 mg stellten sich schon Krämpfe ein.

#### CARDIAZOL

Das Cardiazol<sup>1</sup> oder Pentamethylentetrazol besitzt eine stimulierende Wirkung auf alle Teile des Zentralnervensystems und erhöht die Reflexreizbarkeit des Rückenmarks. In toxischen Dosen ruft es typische epileptiforme Krämpfe hervor.

Die analeptische, wahrscheinlich direkt das vasomotorische und Atmungszentrum betreffende Wirkung des Cardiazols zeigt sich besonders nach zentraler Depression durch Hypnotica. Es erhöht das Volumen und die Frequenz der Atmung, besonders aber die Tiefe des einzelnen Atemzugs (10, 12). Seine antagonistische Wirkung gegen Narcotica ist beträchtlich, und es gilt immer noch neben dem Pikrotoxin als eines der wichtigsten Medikamente bei der Behandlung von Schlafmittelvergiftungen (11).

Die Wirkung des Cardiazols ist in insgesamt 10 Versuchen untersucht worden. Die intravenöse Morphinosis, 4 mg/kg, war in

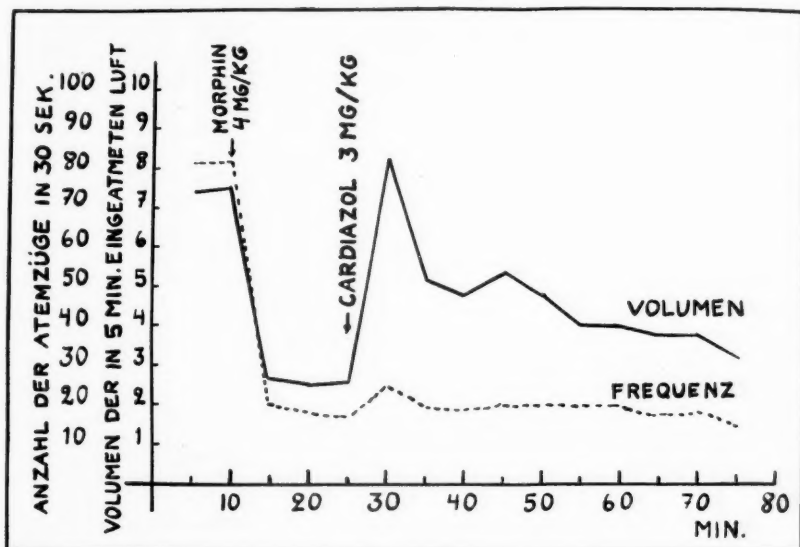


Abb. 2. — Wirkung des Cardiazols auf die durch Morphin deprimierte Atmung beim Kaninchen. Gewicht des Versuchstiers 3.1 kg. Morphinhydrochlorid 4 mg/kg intravenös und danach Cardiazol 3 mg/kg intravenös.

<sup>1</sup> Knoll & Co. A.G., Liestal.

allen Versuchen gleich. Das Cardiazol wurde intravenös in Dosen von 2—7 mg/kg verabreicht. Bei Dosen von 2—4 mg stieg das Atmungsvolumen deutlich über das durch Morphin herabgesetzte Niveau, erreichte aber nur in einem von vier Versuchen sein normales Ausmass (Abb. 2). Mit Dosen von 5—7 mg wurde eine kräftige Wirkung erzielt, wobei das Atmungsvolumen den Normalwert beträchtlich überstieg. Diese Dosen waren schon so gross, dass die Versuchstiere unruhig wurden, und bei den grössten Gaben stellten sich Krämpfe ein. Die Atmungsfrequenz nahm etwas zu, aber in erster Linie wurde die Atmung tiefer. Je nach der Dosis hielt die Wirkung 10—15 Minuten lang an.

#### DAS NEOSPIRAN

Das Neospiran<sup>1</sup> oder Orthophthalsäurebisdiäthylamid besitzt eine starke zentrale Wiederbelebungs Wirkung. Es gilt ein kräftiges Atmungsstimulans, das auch die Zirkulation günstig beeinflusst (6). In Tierversuchen hat es die durch Narcotica herabgesetzte Atmung und den Blutdruck auf das ursprüngliche Niveau zu bringen vermocht. Die Wirkung ist ziemlich anhaltend (6, 1). Das Neospiran beeinflusst direkt das Gefäss- und Atmungszentrum. Die Wirkung stellt sich in voller Intensität ein, auch wenn der Carotissinus beiderseits denerviert ist. Die Weckwirkung ist schwächer als beim Pentamethylentetrazol, und die für diese Weckwirkung erforderlichen Dosen rufen schon Krämpfe hervor (1). In der klinischen Praxis sind keine nachteiligen Nebenwirkungen beobachtet worden. Nach Boecker (2) ist der Stoff in manchen chirurgischen Fällen, bei denen zentrale Analeptica benötigt werden, vorteilhaft. Baetner (3) empfiehlt den Stoff bei akuten Zirkulations- und Atmungskollapsen, wie bei Ertrinkungs- und Elektrizitätsunfällen, Narkosekomplikationen, Arznei- und Leuchtgasvergiftungen. Subkutan werden 2—3-fache Dosen und peroral 10—20-fache benötigt, um den gleichen Effekt wie bei intravenöser Verabreichung zu erzielen (6).

In unseren Untersuchungen haben wir 13 Versuche über die Wirkung des Neospirans auf die durch Morphin deprimierte Atmung beim Kaninchen ausgeführt.

In zwei Versuchen wurde 0.5 mg/kg Neospiran intravenös verabreicht. Beide ergaben Steigerung des Atmungsvolumens, und in

<sup>1</sup> Chemische Fabrik Grünau A.G., Berlin—Grünau.

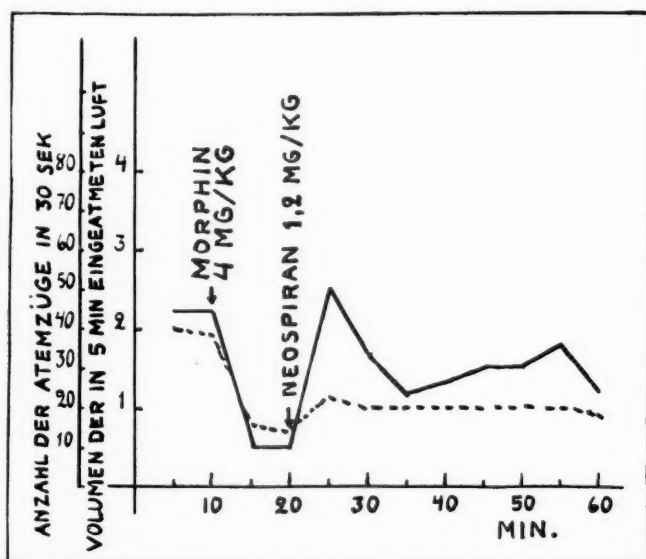


Abb. 3. — Wirkung des Neospirans auf die durch Morphin verminderte Atmung des Kaninchens. Gewicht des Versuchstiers 2.6 kg. Morphinhydrochlorid 4 mg/kg intravenös und danach Neospiran 1.2 mg/kg intravenös.

dem einen Versuch überstieg dasselbe den normalen Wert (Abb. 3). Die Atmungsfrequenz nahm unter dem Einfluss des Neospirans hier nicht wesentlich zu.

In fünf Versuchen injizierten wir intravenös 1 mg/kg Neospiran. Die durch Morphin herabgesetzte Atmung stieg bei allen diesen Tieren wieder auf den normalen Wert, und in einem Versuch nahm das Atmungsvolumen über das normale Ausmass zu. In zwei weiteren Versuchen, bei denen dem einen Versuchstier 1.5 mg und dem anderen 2 mg/kg Neospiran injiziert wurde, stieg das Atmungsvolumen auf den normalen Wert und etwas darüber. Die Dosis von 2 mg rief bei den Tieren Unruhe und Zuckungen hervor.

Als wir 3 mg/kg und noch grössere Dosen verabreichten, wurde die Wirkung des Neospirans nicht wesentlich besser. Der Effekt trat zwar schneller ein, hielt aber kürzere Zeit an, und am nächsten Tag wurde bei allen Tieren, die 3 mg/kg oder mehr Neospiran bekommen hatten, Lähmung des Hinterkörpers sowie Incontinentia ani et urinae festgestellt. Diese Erscheinungen waren irreparabel. Bei keinem anderen von uns untersuchten Stoff haben wir derartige Wirkungen beobachtet. Im allgemeinen war die mässige

Wirkung des Neospirans in unseren Versuchen anhaltend, indem sie 15—40 Minuten lang dauerte, und auch dann war das Atmungsvolumen noch nicht ganz auf das durch Morphin herabgesetzte Niveau gesunken.

#### DAS CYCLITON

Das Cycliton<sup>1</sup> ist ein Oxazolderivat, 3.5-Dimethyl-Isoxazol-4-Carbonsäurediäthylamid, das hinsichtlich seiner chemischen und pharmakologischen Eigenschaften an das Coramin erinnert. In der klinischen Medizin wird es als Atmungs- und Zirkulationsstimulans angewandt. Es verursacht etwas leichter Krämpfe als das Coramin, jedoch nicht so leicht wie das Cardiazol (7). Experimentell ist festgestellt worden, dass das Cycliton die durch Morphin verminderte Atmung des Kaninchens auf das normale Niveau zu steigern vermag (8).

Den Einfluss des Cyclitons auf die durch Morphin deprimierte

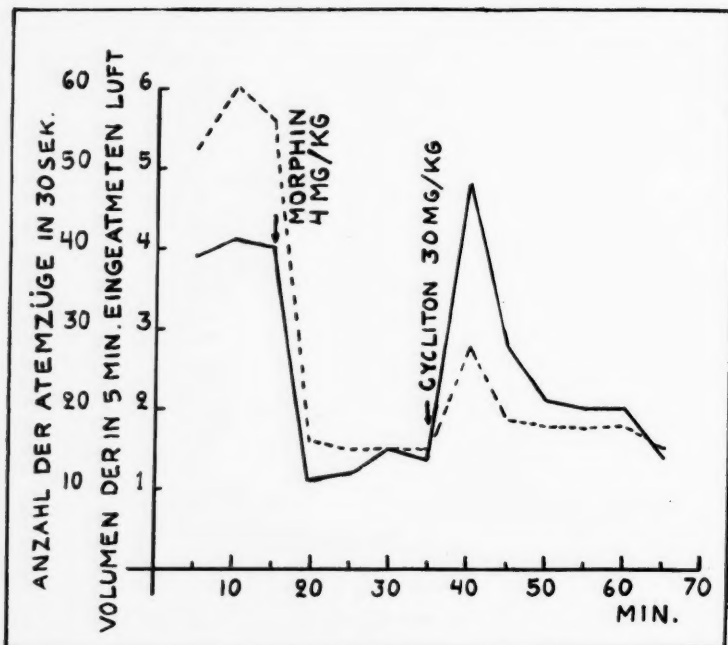


Abb. 4. — Wirkung des Cyclitons auf die durch Morphin deprimierte Atmung beim Kaninchen. Gewicht des Versuchstiers 2.4 kg. Morphinhydrochlorid 4 mg/kg intravenös und danach Cycliton 30 mg/kg intravenös.

<sup>1</sup> F. Hoffmann-La Roche & Co. A.G., Basel.

Atmung des Kaninchens haben wir in 10 Versuchen untersucht. Den Versuchstieren wurden intravenöse Gaben Cycliton von 20—60 mg/kg verabreicht.

Mit einer intravenösen Dosis von 20 mg/kg wurde eine leichte, aber doch deutlich wahrnehmbare Erhöhung des Atmungsvolumens erzielt, die nur kurze Zeit, 5—10 Minuten lang, anhielt.

In zwei Versuchen verabreichten wir 30 mg/kg Cycliton intravenös, wonach das Atmungsvolumen deutlich stieg und ungefähr das normale Ausmass erreichte, welche Wirkung 10—30 Min. lang anhielt. Auch die Atmungsfrequenz nahm etwas zu. Bei zwei weiteren Versuchen wurde mit Dosen von 40 mg/kg eine ähnliche Wirkung erzielt.

In desgleichen zwei Versuchen erhielten die Kaninchen 50 mg/kg Cycliton intravenös, und die Wirkung war kräftig sowie ziemlich anhaltend, nämlich ca. 20—30 Min. Die Atmungsfrequenz stieg deutlich.

Schliesslich wurde in zwei Versuchen den Tieren 60 mg/kg in die Vene injiziert. Bei beiden nahm das Atmungsvolumen deutlich über den normalen Wert zu, und entsprechend stieg die Atmungsfrequenz. Das eine Kaninchen bekam schon deutliche Krämpfe, während das andere sich ruhig verhielt.

#### DAS HEXETON

Hexeton<sup>1</sup> oder Methylisopropylzyklohexanon ist mit Kampfer isomer. Das Hexeton ist eine wasserklare Flüssigkeit, die als Natriumsalizylat wasserlöslich ist. Hinsichtlich seiner pharmakologischen Eigenschaften ist es dem Kampfer sehr ähnlich. Nach den Untersuchungen von Kohn und Jacobi (9) hat es jedoch in grossen Dosen eine erhebliche lähmende Wirkung, und Krämpfe treten erst kurz vor dem Tode auf. Ihrer Auffassung gemäss besitzt das Hexeton eine gute Weckwirkung, und der gleichen Ansicht ist auch Gremels (4). Zipf und Mertins (15) hingegen erachten die antinarkotische Wirkung für schwach.

Den Einfluss des Hexetons auf die durch Morphin herabgesetzte Atmung des Kaninchen haben wir in 10 Versuchen untersucht, wobei wir Dosen von 2—10 mg/kg verabreichten. Mit einer Gabe von 1 mg/kg erhielten wir eine eben wahrzunehmende Steigerung des

<sup>1</sup> »Bayer« Igepha A.G., Zürich.

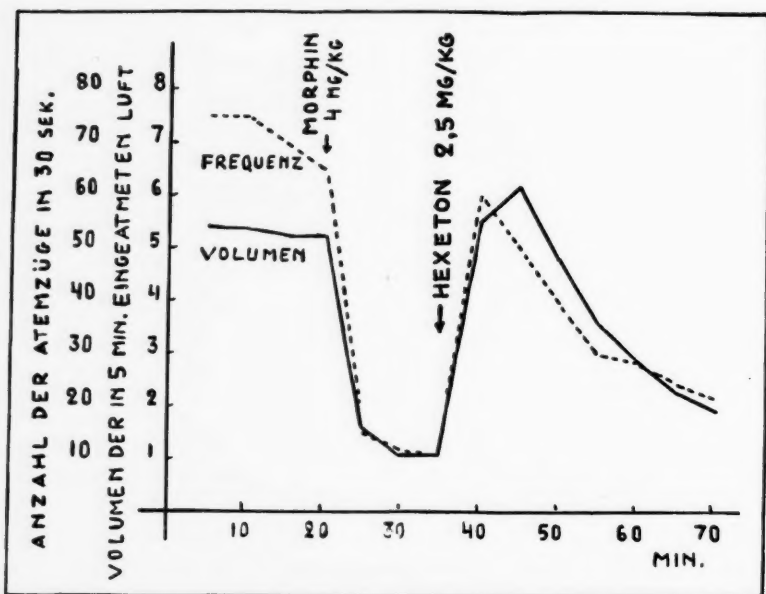


Abb. 5. — Wirkung des Hexetons auf die durch Morphin herabgesetzte Atmung des Kaninchens. Gewicht des Versuchstiers 2.5 kg. Morphinhydrochlorid 4 mg/kg intravenös und danach Hexeton 2.5 mg/kg intravenös.

Atmung, mit 2 mg/kg in vier Versuchen nahm die Atmung deutlich zu, obschon das Atmungsvolumen noch nicht auf das normale Niveau stieg. Bei einer Dosis von 2.5 mg/kg überstieg das Atmungsvolumen schon den normalen Wert, aber das Versuchstier bekam für einen kurzen Augenblick leichte Krämpfe.

Nach Verabreichung von 3 mg/kg stieg das Atmungsvolumen auf die normale Höhe, das Versuchstier war ein paar Minuten lang erregt und beruhigte sich dann. Wenn 4 mg/kg gegeben wurden, bekam das Kaninchen Krämpfe, und das Atmungsvolumen stieg beträchtlich über den normalen Umfang, wobei die Atmung unregelmässig war. Bei 5 mg/kg stieg das Atmungsvolumen über den normalen Wert, und es stellten sich zeitweilige Zuckungen ein.

In unseren Versuchen war die Wirkung des Hexetons ganz ähnlich, wie wir früher mit der gleichen Versuchsanordnung bezüglich des Kampfers und des damit isomeren Methylsantenons festgestellt hatten (13).

TABELLE 1

Unter- suchter Stoff	Intravenöse Dosis mg/kg				Dauer der Wirkung	Stimula- tionsbreite koeffizient
	Schwache Wirkung	Mittel- mässige Wirkung	Kräftige Wirkung	Krämpfe verursach- ende Dosis		
Strychnin	0.05			0.07	20—60	0
Pikrotoxin		0.15	0.2	0.25	30—90	1.7
Lobelin ..	0.5	1.0	1.5	2.5	5—10	2.5
Koffein ..	40.0	50.0	60.0	65.0	20—60	1.3
Pervitin ..	2.0	2.0	3.0	6.0	30—90	3.0
Coramin ..	10.0	15.0	20.0	30.0	10—25	2.0
Cardiazol	2.0	3.5	4.5	5.0	10—15	1.4
Neospiran	0.5	1.0	1.5	2.0	15—40	2.0
Cycliton ..	20.0	30.0	50.0	60.0	5—30	2.0
Hexeton ..	1.0	2.0	2.5	3.0	5—20	1.5

## DISKUSSION UND SCHLUSSFOLGERUNGEN

In der Tabelle Nr. 1 haben wir die Resultate unserer Versuche zusammengestellt. Als schwache Wirkung bezeichnen wir leichte, aber doch deutlich wahrzunehmende Steigerung der durch Morphin deprimierten Atmung. Mittelmässige Wirkung bedeutet Ansteigen des durch Morphin verminderten Atmungsvolumens bis nahe zu den normalen Werten. Unter kräftiger Wirkung verstehen wir Erhöhung des herabgesetzten Atmungsvolumens auf das normale Niveau oder darüber. Die Zahlen in der Tabelle entsprechen der aus den Versuchsergebnissen berechneten Wahrscheinlichkeit.

In unseren Versuchen haben wir auch die Veränderungen der Atmungsfrequenz untersucht. Die technische Anordnung des Versuchs gestattet dem Versuchstier keine sehr grossen Veränderungen der Frequenz. Der Widerstand des Gasometers und der Klappen verursacht sog. Gasmaskenatmung. In gleicher Richtung wirkt auch die in den Röhren hin und her gleitende Luftsäule. Wir hatten versucht, beide Faktoren in unseren Versuchen auf ein Minimum herabzusetzen. In den meisten Fällen war die Zunahme des Atmungsvolumens durch eine Vertiefung der Atemzüge bedingt, während die Atmungsfrequenz fast unverändert blieb. Am deutlichsten nahm die Frequenz nach der Verabreichung von Lobelin, Cycliton, Koffein und Hexeton zu. Auch das Geschwindigkeit beim Einspritzen, das wir in unseren Versuchen konstant zu halten suchten, hat seinen Einfluss auf die Versuchsergebnisse.

Um den gegenseitigen Vergleich der zu untersuchenden Stoffe zu erleichtern, haben wir den sogenannten Stimulationsbreitkoeffizient berechnet, der das Verhältnis zwischen der krämpfeverursachenden und der mittelmässige Wirkung erzeugenden Dosis ist. Wie aus der Tabelle hervorgeht, gehören die Zahlen im grossen und ganzen zur gleichen Grössenklasse. Beim Strychnin ist der Stimulationsbreitkoeffizient 0, weil mit diesem Stoff in den Versuchen nicht einmal mässige Wirkung erzielt werden konnte. Das Pervitin hat einen etwas grösseren Stimulationsbreitkoeffizient als die anderen Stoffe, aber die Atmung war in vielen Pervitinversuchen periodisch. Da bei allen Stoffen, mit Ausnahme des Lobelins, die Wirkungsweise vom gleichen Typus sein dürfte, ist es verständlich, dass auch der Effekt und die Stimulationsbreitkoeffizienten hinsichtlich ihrer Grösse nahe beieinander liegen. Zur Erzielung einer effektiven Wirkung muss die Atmung mit Dosen stimuliert werden, die den toxischen Gaben recht nahe kommen. Wie aus der Tabelle ersichtlich ist, schwankt die Dauer der Wirkung beträchtlicher als der Stimulationsbreitkoeffizient, aber bei allen verglichenen Stoffen ist sie doch ziemlich kurz.

#### ZUSAMMENFASSUNG

In der Untersuchung ist eine Serie von vergleichenden Versuchen über die Wirkung einiger zur Gruppe der zentralen Analeptica gehöriger Stoffe auf die durch Morphin deprimierte Atmung beim Kaninchen angestellt worden. Zur Erleichterung des Vergleichs wurde der sog. Stimulationsbreitkoeffizient berechnet, womit das Verhältnis zwischen der krämpfeverursachenden Dosis und der mässigen, die Atmung ungefähr auf den normalen Wert steigernden Dosis gemeint ist. Die Koeffizienten waren ungefähr von der gleichen Grössenklasse. Die Dauer der Wirkung war bei den untersuchten Stoffen weit mehr verschieden als die Stimulationsbreitkoeffizienten, und verhältnismässig kurz. Zur Erzielung einer effektiven Wirkung musste die durch Morphin deprimierte Atmung mit Dosen stimuliert werden, die den toxischen Gaben nahe kamen.

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## ROUTINE CULTURE FOR TRICHOMONAS AND YEAST-LIKE FUNGI

by

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(Received for publication April 5, 1954)

Penttinen and Rauramo (3) and Larjanko (2) were the first in Finland to publish results concerning trichomonas culture. The series of cases of the former investigators consisted of 127, that of the latter 100 patients. In the Department of Serology and Bacteriology, University of Helsinki, routine culture for trichomonas and yeast-like fungi has been carried out since Febr. 1, 1948, Halonen and Pättiälä (1) published the results obtained until the year 1950. The results obtained until the end of 1952 are reported in the present paper.

### MATERIAL

The series consisted of 10,240 cases from various hospitals in Helsinki, from maternity centres, private practitioners and from private laboratories in Helsinki and Lahti. The culture was mostly made from the vaginal or cervical secretion, only in a few cases it was made from the urine. The series of cases included only 20—30 males a year. Due to the minority of the two latter groups they have not been discussed separately.

### TECHNIQUE

The cultivation was performed in test tubes which contained 2 ml of culture medium of the following composition:

Peptone (Witte) .....	32 g
Agar .....	1.6 »
1(-) cysteine hydrochloride .....	2.4 »
Maltose .....	1.6 »
Liver infusion (A mixture of one part liver and two parts water) .....	320 ml
Ringer's solution .....	960 »
Methylene blue (0.5 per cent) .....	0.7 »
The medium was sterilized and adjusted to pH 6 with NaOH.	
Human serum .....	320 »
Penicillin .....	1,600,000 units
were added.	

The tubes were incubated at 37° C. The first reading was performed after 24, the second after 48 hours, by microscoping a drop of the medium on a slide.

## RESULTS

Out of the 10,240 specimens examined 2,255 (22 per cent) proved trichomonas-positive, 2,239 (21.9 per cent) yeast-positive, and 307 (3 per cent) both trichomonas- and yeast-positive. In 4,494 cases the sender of the specimen had performed immediate microscopy of the native preparation for trichomonas. Table 1 shows the relation between the results obtained by immediate microscopy and by cultivation.

TABLE 1

COMPARISON BETWEEN THE RESULTS OBTAINED BY IMMEDIATE MICROSCOPY OF  
THE NATIVE PREPARATION AND BY CULTIVATION

Year	Micr. + (+?) Cult. +	Micr. + (+?) Cult. —	Micr. — (—?) Cult. +	Micr. — (—?) Cult. —	Total
1948	32	10	40	300	382
1949	58	19	70	687	834
1950	42	26	146	969	1183
1951	61	33	148	871	1113
1952	63	45	72	805	985
Total	256	133	476	3632	4497

The seasonal distribution of positive trichomonas- and yeast-like fungi-specimens is to be seen in Table 2.

TABLE 2  
SEASONAL DISTRIBUTION OF THE POSITIVE CASES WITH TRICHOMONAS AND YEAST-LIKE FUNGI IN THE YEARS 1948—1952

Month	Cases	Trichomonas +		Yeast +		Trichomonas and Yeast +
		Number	Per Cent	Number	Per Cent	
I	770	106	4.7	149	6.6	12
II	793	179	8.0	132	5.9	21
III	764	193	8.6	150	6.7	20
IV	819	191	8.5	168	7.5	22
V	903	195	8.6	237	10.6	36
VI	978	235	10.4	226	10.1	32
VII	655	131	5.8	138	6.2	18
VIII	704	178	7.9	172	7.7	24
IX	1005	263	11.7	201	9.0	35
X	1041	195	8.6	227	10.1	28
XI	1042	235	10.4	227	10.1	30
XII	766	154	6.8	212	9.5	29
Total	10240	2255	100.0	2239	100.0	307

TABLE 3  
AGE DISTRIBUTION OF THE POSITIVE CASES WITH TRICHOMONAS AND YEAST-LIKE FUNGI IN THE YEARS 1951—1952

Age in Years	Trichomonas +		Yeast +	
	Number	Per Cent	Number	Per Cent
10—15	1	0.3	4	1.0
16—20	7	2.0	8	2.0
21—25	48	13.8	61	15.3
26—30	73	21.1	104	26.1
31—35	67	19.3	80	20.0
36—40	65	18.7	65	16.3
41—45	40	11.5	42	10.5
46—50	34	9.8	23	5.8
51—55	10	2.9	6	1.5
56—60	1	0.3	5	1.3
61—65	—	—	—	—
66—70	1	0.3	1	0.2
Total	347	100.0	399	100.0

During the years 1951—52, out of the cases in which the age of the patient was known, 347 were trichomonas-positive and 399 were yeast-positive. Table 3 shows their distribution into various age groups.

The distribution of positive cases seems to correspond to the relative sexual activity of the age groups.

#### DISCUSSION

References in the earlier literature (4) indicate that the cultivation and the immediate microscopy of native preparation reveal approximately the same amount of positive results, provided that the latter is performed carefully enough. In the everyday routine work, however, the cultivation is obviously more reliable. Thus, in our material (Table 1) the ratio of positive results obtained by these methods is 732 : 389, or about 2 : 1, in favour of the cultivation. On the other hand, in 133 cases the cultivation revealed negative results though trichomonas was found by immediate microscopy of the native preparation. A part of these may erroneously have been taken positive (especially reading +?). Besides, the unfavourable transportation conditions of the test tubes may have caused the death of trichomonas. There is also a possibility that «overgrowth» of bacteria had occurred in the culture medium in spite of penicillin. It seems likely, then, that the immediate microscopy of the native preparation is of significance in the routine-like trichomonas diagnostics as an additional method owing to its rapidity and reliability of the results.

When comparing the seasonal incidence of trichomonas and yeast-like fungi (Table 2) it is to be seen that the former group seems to reach its maximum in autumn, the latter in May and June, while the both groups are in their minimum in winter. The decrease of the both groups in July and August may be due to the smaller amount of specimens sent to our Laboratory because of the summer holidays.

When studying the amount of trichomonas-positive cases, they are noted to correspond to the relative sexual activity of the various age groups. This agrees with the general opinion (4) that among the principal means of conveyance the sexual intercourse is of importance. This is also the case with yeast-like fungi. How-

ever, it is to be noted that in the years 1951—52 chlortetracycline (aureomycin) and oxytetracycline (terramycin), which increase the growth of yeast-like fungi in vagina, were largely used therapeutic agents in trichomonas vaginitis. This may have influenced on the corresponding distribution of yeast-like fungi in various age groups, too.

#### SUMMARY

1. Out of the 10,240 specimens, taken mostly from vagina and cervix and only in a few cases from the urine, trichomonas were identified in the routine culture in 2,255 cases (22 per cent), yeast-like fungi in 2,239 cases (21.9 per cent) and both of them in 307 cases (3 per cent).

2. Compared with the immediate microscopy of the native preparation the cultivation for trichomonas gave twice as many positive results.

3. The incidence of trichomonas was highest in autumn, that of yeast-like fungi in May and June. The total incidence was lowest in winter.

4. The distribution of positive cases with trichomonas was approximately corresponding to the relative sexual activity of the age groups. This is also the case with yeast-like fungi, though it may be partly due to the antibiotics used.

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# BARBAMYL

hypnoticum —  
sedativum



# INDEX

Vol. 32. Fasc. 2.

	Pag.
KIMMO K. MUSTAKALLIO and ANTTI TELKKÄ (Helsinki): Effects of Mercurial Diuretics in Rat Kidney .....	123
KARI PENTTINEN and VEIKKO TOMMILA (Helsinki): The Capacity of Small Amounts of Influenza Virus to Destroy Egg Mucin Inhibitor .....	134
N. OKER-BLOM and OLOF ALFTHAN (Helsinki): Transplantation of Human Normal and Neoplastic Tissue to the Chorio Allantoic Membrane of Embryonated Chicken Eggs .....	143
TERTTU ARAJÄRVI (Helsinki): Aether Anaesthesia: Influence on the Formation of Intravascular Red Cell Aggregation in Children ..	160
TERTTU ARAJÄRVI and G. R. WALLGREN (Helsinki): Local Effect of Aether and Other Anaesthetics on the Conjunctival Blood Vessels and Their Circulating Red Cells .....	168
KIMMO K. MUSTAKALLIO (Helsinki): Histochemical Demonstration of Succinic Dehydrogenase Activity in Human Brain .....	175
EEVA JALAVISTO (Helsinki): Plasma Oxidation-Reduction Potential and the Hypoxic Stimulus of Blood Formation .....	178
ESKO K. NÄÄTÄNEN (Helsinki): The Paradoxical Effect of Giant Intravenous Insulin Doses on Rabbits .....	186
EEVA JALAVISTO (Helsinki): The Effect of Ephynal on the Post-haemorrhagic Reticulocytosis and Regeneration of Blood in Rabbits .....	195
EINO V. VENHO, OSMO VARTIAINEN und MATTI VAPAAVUORI (Helsinki): Vergleichende Versuche über die Wirkung verschiedener Atmungsstimulansen, II Teil .....	203
R. PÄTIÄLÄ and E. J. JOKINEN (Helsinki): Routine Culture for Trichomonas and Yeast-like Fungi .....	214